MECHANISMS OF CARBARYL RESISTANCE IN THE FALL ARMYWORM, *Spodoptera frugiperda*

BY

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1985

I Elzie McCord, Jr., dedicate this dissertation to:

- my family, Pinkie W., wife, Rogers Christopher and Timothy Ryan, sons, for their continued support, understanding and companionship, and
- Ms. Lue Vester Davis for being an ideal role model, for forfeiting her one free hour during the school day to teach a select few of us the slide rule, basic and advanced algebra and trigonometry, for coercing parents to impress upon their children the importance of performing well in school and for inspiring my career in the biological sciences.

ACKNOWLEDGEMENTS

Since it is nearly impossible to thank everyone who has contributed to the success of my career, this research and the preparation of this manuscript, I will attempt to mention those who have contributed the most without diminishing the roles of those not named here.

I wish to thank the following people:

o My parents, Pearlence C. and Elzie, Sr., for their continued encouragement and support in my pursuit of personal goals and for guiding me in a direction that made goal selection possible,

o Dr. S. H. Kerr, for supporting me throughout my pre- and post-graduate studies at the University of Florida,

o Dr. Simon S. J. Yu for allowing me the opportunity to study and work with him under somewhat accelerated conditions; for providing encouragement, constructive criticism, and assistance in performing the complex biochemical processes for which he is highly regarded, and for his belief in me and my abilities to accomplish the program described herein,

o Drs. J. L. Nation, J. R. Strayer, D. L. Shankland, and R. B. Shireman for quality educationsl instructions, for serving on my supervisory committee and for reviewing this manuscript,

o Mr. and Mrs. Siegfried J. Schulze, Mr. Willie Foresto, Ms. Alice Foresto and Mr. Lutz Schulze for being such wonderful neighbors who took care of my family while I completed the research herein,

o Dr. J. R. Young, USDA, Agricultural Research Center, Tifton, Georgia, for inspiring this research project and for providing me with the resistant fall armyworm strain,

o Drs. A. B. Meade, K. S. Amuti, Der-I Wang, T. M. Priester, Ms. Babirette Babineaux, Ms. C. N. Selz, Mr. Matthew McGirr and others who have contributed moral support, ideas, constructive criticisms, drawings, computer wizardry, etc.,

o The Agricultural Chemicals Department of the E. I. Du Pont de Nemours & Company, Drs. Dale Wolf, K. A. Saegebarth, G. D. Hill, H. M. Loux, J. W. Searcy, E. J. Soboczenski and others who assisted in my leave of absence request and those who approved the granting of that request,

o Mr. Philip N. Chaney and Mr. J. L. Jenkins for being the true friends I always wanted and needed,

o Mrs. Jo Ann Ledford and Mrs. Glinda Burnett for their continued friendship and assistance since 1973, and

o Last but not least, my wife Pinkie W. and my sons Rogers Christopher and Timothy Ryan for enduring the hardships of daily activities without me for 18 months, for tolerating my absence while in Florida and while I was locked away in my home office.

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase enzyme
ATC	Acetylthiocholine
BHC	Benzene hexachloride (See HCH)
BSA	Bouine serum albumin
CPB	Colorado potato beetle
DBLS	Diazoblue laurylsulfate
DCNB	1,2-dichloro-4-nitrobenzene
DDT	p,p' dichloro-diphenyl trichloroethane
DEF	S,S,S-tributyl phosphorotrithioate
DFP-ase	Phosphotriester hydrolase
DMC	bis-(p-chlorophenyl) methyl carbinol
DTNB	5,5-dithiobis-2-nitrobenzoic acid
FAW	Fall armyworm
GSH	Glutathione
HCH	Hexachlorohexane (see BHC)
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IBP	S-benzyl 0,0-disopropyl phosphorothioate
Kdr	Knockdown resistance
K_i	Inhibition constant
K_m	Binding affinity
MFO	Microsomal mixed-function oxidase
α-NA	α-naphthylacetate
β-NA	β-naphthylacetate

NADPH	Nicotinamide adenine dinucleotide phosphate
O.D.	Optical density
OP	Organophosphate insecticide
p-NPA	p-Nitrophenyl acetate
PB	Piperonyl butoxide
PCA	p-chloroaniline
PCMA	p-chloro-N-methylaniline
PCMB	p-chloromercuribenzoate
PDAB	p-dimethylaminobenzaldehyde
PHMB	p-hydroxymercuribenzoate
R	Resistant insect strain
R-AChE	Acetylcholinesterase enzyme from resistant strain
R-V_{max}	Maximum reaction velocity of resistant strain
S	Susceptible insect strain
S-AChE	Acetylcholinesterase enzyme from susceptible strain
S-V_{max}	Maximum reaction velocity of susceptible strain
TLC	Thin layer chromatography
TOCP	Tri-creosyl phosphate
TPP	Triphenyl phosphate
USDA	United States Department of Agriculture
V_{max}	Maximum reaction velocity
WHO	World Health Organization

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of the University of Florida in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

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August, 1985

Chairman: Dr. S. J. Yu
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Mechanisms of resistance to carbaryl were investigated in larvae of the fall armyworm, Spodoptera frugiperda (J. E. Smith). Piperonyl butoxide greatly reduced the resistance ratio from > 90-fold to 6-fold suggesting the involvement of microsomal cytochrome P-450-dependent monooxygenases. In vitro metabolic studies revealed that oxidative metabolism of carbaryl by midgut microsomes was 5 times more active in the resistant strain compared to the susceptible strain. In addition, activities of midgut microsomal epoxidase and hydroxylase were significantly higher during the larval stage in the resistant strain than in the susceptible strain.

Cuticular penetration studies using [^{14}C] carbaryl showed that 60% of the applied radioactivity remained on the cuticle of resistant larvae while 32% remained on susceptible larvae 24 hr after topical treatment. There was no difference in the amount of radioactivity found internally in the two strains. Susceptible larvae, however, excreted 4 times more

radioactivity than resistant larvae. It is concluded that enhanced oxidative metabolism of carbaryl plays an important role in the carbaryl resistance. Slower penetration of carbaryl in the resistant armyworm may be a minor factor contributing to resistance.

INTRODUCTION

Resistance has been defined as "the developed ability in a strain of insect to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species" (Anonymous 1957). It is preadaptive in nature, representing a selection of genes already present in the population. As susceptible individuals are killed from the selected population, resistant individuals breed and pass resistance genes to their progeny. The continued use of the same or similar insecticides increases the selection pressure on the population and causes resistance expression in the majority of the individuals in that population. Georghiou and Mellon (1983) reported that a total of 428 insect and acarina species were resistant to one or more insecticide classes including those commonly used today. These insecticide classes include DDT-analogues, cyclodiene/BHC, organophosphates (OP's), carbamates, insect growth regulators, pyrethrins and the newer synthetic pyrethroids (Priester 1979; Wolfenbarger et al. 1981; Sparks 1980; Bull 1981; Brown 1981).

Insect resistance to insecticides can be divided into two types, behavioral resistance and physiological resistance. Recent evidence shows that both types of resistance often coexist in resistant individuals (Lockwood et al. 1984). Behavioral resistance is mostly stimulus dependent, requiring sensory stimulation to achieve avoidance. Insects with behavioral resistance are more sensitive and are able to respond to lower concentrations of insecticides than are susceptible insects.

There are three main types of physiological resistance, namely, increased detoxication, reduced penetration, and target site insensitivity.

Increased insecticide metabolism by specific detoxication enzymes was found to confer carbamate, organophosphate and/or chlorinated hydrocarbon resistance in numerous species of insects (Hughes 1982; Yu and Terriere 1979; Motoyama et al. 1980; Kuhr 1970; Kao et al. 1984; Devonshire and Moores 1982; Clark et al. 1984; Rose and Sparks 1984; Plapp 1970; Wool et al. 1982).

Reduced penetration as a resistance mechanism was reported in several insect species (Eldefrawi and Hoskins 1961; Ku and Bishop 1967; Hanna and Atallah 1971; Ahmad et al. 1980; Ariaratnam and Georghiou 1975; Patil and Guthrie 1979; Sinchaisri et al. 1978).

Target site insensitivity, including insensitive acetylcholinesterase as a resistance mechanism was reported in several insect species (Roulston et al. 1968 and 1969; Iwata and Hama 1972; Hama and Iwata 1971 and 1978; Devonshire 1975; Yamaoto et al. 1977; DeVries and Georghiou 1981a and 1981b; Yeoh et al. 1981; Devonshire and Moores 1984).

Carbaryl (1-Naphthyl-N-methylcarbamate), a reversible cholinesterase inhibitor, is an agricultural pesticide used in the control of over 150 major pests (Mount and Oehme 1981). Carbaryl is safe to mammals, having an acute oral LD_{50} greater than 500 mg/kg body weight in rats (Mount and Oehme 1981; Terriere 1982). It is short lived in the environment. However, its high toxicity to honey bees has restricted its use on some highly pollinator dependent crop plants, and has limited its time of application on others. The apparent success of carbaryl since its introduction in 1956 has been due to its reliability of control, safety to humans and wildlife, and the array of insects controlled.

The fall armyworm, Spodoptera frugiperda (J. E. Smith), is a voracious phytophagous insect pest of the southeastern U.S. and the tropics (Luginbill 1928; Vickery 1929). The fall armyworm (FAW) damages many crop plants by feeding on leaves and fruit, often consuming the entire leaf, except the mid-rib, or producing holes in the leaves (Vickery 1929) as a result of sporadic feeding.

Young and McMillian (1979) reported that FAW had become resistant to carbaryl insecticide but remained susceptible to a related carbamate insecticide, methomyl. FAW resistance to the organophosphates, trichlorfon, diazinon, methyl parathion and parathion was reported by Bass (1978).

It is important to study the mechanisms of resistance in order to better understand how to slow down or lessen the severity of widespread insect resistance to insecticides. The research reported here was designed to investigate resistance mechanisms in a field collected resistant strain of FAW.

Specific objectives were to determine the following:

1. The susceptibility of the field collected strain to related carbamates, organophosphorous and synthetic pyrethroid insecticides as compared to a susceptible laboratory strain.

2. The activities of various detoxication enzymes in the resistant and susceptible strains.

3. The differences in the rate of cuticular penetration of carbaryl in both strains.

LITERATURE REVIEW

Status of Resistance

Insect resistance to insecticides has been known since the early 1900s. Melander reported San Jose scale resistance to lime-sulfur in 1914 and Quayle reported resistance in the California red scale to cyanide in 1916 (O'Brien 1967; Forgash 1984). Since these early reports, resistance has been reported in organisms other than insects such as bacteria, sporozoa and mammals (Georghiou and Mellon 1983). Nowhere has the impact of organisms expressing resistance been as great as with insects. Georghiou and Mellon (1983) reported, conservatively, 428 known insect and acarina species world-wide that have developed resistance. Georghiou (1980) emphasized that the number of resistant insect species is not as staggering as the number of chemicals that many insect strains can now tolerate and the increased geographical distribution of resistant insect populations.

The wide distribution of resistant species suggests a common phenomenon called cross-resistance which allows one organism to become resistant to insecticides of the same and different classes due to the same resistant mechanism (Oppenoorth and Welling 1976). Priester (1979) reported cross-resistance in Culex quinquefasciatus Say to synthetic pyrethroids with implication of prior DDT exposure. Scott et al. (1983) reported cross-resistance in six predatory mite strains to permethrin that also had previous exposure to DDT, azinphosmethyl, parathion and carbaryl.

Resistance that is related to previous exposure suggests a genetic change that influences massive physiological and biochemical changes in an organism. Plapp (1984, p. 194) states that "it is becoming apparent that changes at only a few loci are responsible for resistance to many insecticides. That is, the genetic basis for resistance is relatively simple. This is why cross-resistance to insecticides is such a severe problem. Selection for resistance to a specific chemical often confers resistance not only to the selecting agent, but sometimes to all insecticides having the same mode of action and other times to virtually all chemicals metabolized by one or more of the major detoxification enzyme systems".

Wolfenbarger et al. (1981) reported geographical locations of resistant Heliothis zea (Boddie), H. virescens (F.), H. armigera (Hubner) and H. puntigera (Wallengren) in Mexico, Central America, South America, Australia, Africa and Asia. Wolfenbarger's survey included countries or continents where each species was indigenous. Sparks (1981) emphasized the severity and importance of resistant Heliothis zea (Boddie) and H. virescens (F.) in North America, concluding that these species are two of the most serious agricultural pests. Bull (1981) noted that H. virescens (F.) had become resistant to many of the older chlorinated hydrocarbon and organophosphorus insecticides and apparently has some cross tolerance to certain of the new synthetic pyrethroids and organophosphorus insecticides recently developed for its control.

Graham-Bryce (1983) concluded that increases in resistance to conventional pesticides require investigation of novel chemical approaches to crop protection. He suggested the investigation of

unexploited target sites, the modification of chemical properties of pesticides to increase mobility and availability, the exploration of novel formulations, and the investigation of chemical compounds that suppress chemically mediated processes rather than functioning by direct toxic action. The approaches suggested by Graham-Bryce (1983) would serve to slow down resistance, produce selective compounds, reduce mammalian toxicity and afford control comparable to more toxic, environmentally persistent compounds now in use.

Genetics of Resistance

The World Health Organization's (WHO) definition of resistance denotes resistance as a property of a population and not the result of alterations within individual insects (Oppenoorth and Welling 1976). It is the individual insect that possesses the preadaptive ability to withstand higher than normal toxic doses of pesticides. Resistance is assumed to be preadaptive arising through recurrent mutation of existing alleles (Sawicki and Denholm 1984). Mutations of genes can be monogenic or polygenic, and those terms are synonymous with mono- and multifactorial, respectively, meaning resistance is under the control of one or several genes. It is not known which mutation will occur under which insecticidal pressure for a given insecticide. However, Oppenoorth and Welling (1976) predict monogenic resistance will occur if a single gene can confer high resistance in an organism. Polygenic resistance is less likely to occur, but may occur in organisms exposed to the selecting agent over long periods of time.

Genetics offer a valuable tool in analysis of resistance (Oppenoorth and Welling 1976). Genetics can aid the separation of different resistance mechanisms that occur simultaneously in a strain. Also continuous environmental selection can provide researchers with rare

mutants that without genetic analysis would not be detected (Oppenoorth 1965).

To use genetics as a tool, researchers have developed various cytogenic techniques whereby marker genes can be located on chromosomes, and these chromosomes mapped to determine specific location of alleles on those chromosomes. Priester (1979) used genetic crosses of Culex quinquefasciatus Say to study inheritance of pyrethroid resistance to isomers of permethrin. Farnham (1973) isolated four genetic resistance factors from the house fly, Musca domestica (L.), to natural pyrethrins and resmethrin. Priester (1979) and Farnham (1973) used bioassay technique to determine the presence or absence of expected resistance genes acquired during crossings.

Farnham (1973) found that the resistance genes carried no markers. He replaced the marked autosomes of a quadruple susceptible strain with unmarked resistance genes in an attempt to associate visible phenotypic characters with resistant characteristics. By crossing and back-crossing progeny from both fly strains, he developed four strains which were visibly distinct and which conferred resistance factors specific for penetration, kdr (knockdown resistance), natural pyrethrin resistance and resistance to synergized pyrethrins. These genes were located on chromosomes 3, 3, 5, and 2, respectively.

Predecessors of the above techniques were performed soon after the discovery of organic insecticide resistance. Lovell and Kearns (1959) selected house flies, Musca domestica (L.), with DDT and DMC (bis-(p-chlorophenyl) methyl carbinol). The amount of DDT-ase present in the fly strain selected with DDT alone was much less than in those selected with DDT and DMC. Subsequent back crosses provided initial clues that

DDT resistance may be governed by a single partially dominant gene which behaved according to simple Mendelian principles.

Georghiou et al. (1961) and Georghiou (1962) selected laboratory house flies with various carbamates and tried to reverse resistance with piperonyl butoxide (PB). They concluded that some unknown factor in the fly was insensitive to PB because resistance could not be eliminated entirely. They also recognized that factors other than those inhibited by PB played a major role in carbamate resistance in highly resistant fly strains.

Plapp and Hoyer (1968a), investigating resistance in the mosquito Culex tarsalis Coquillet and the house fly, found that a kdr gene for DDT resistance also conferred resistance to DDT analogues and pyrethrins + PB. By crossing groups of individuals in both species and using discriminating insecticidal doses to isolate the desirable genotypes, Plapp and Hoyer relocated unmarked genes in individuals with phenotypic marker. No metabolic differences were found in the Resistant (R) or Susceptible (S) strain of the mosquito or house fly that could explain the high degree of resistance found to DDT and pyrethrin. This experiment showed that resistance could occur without the presence of high levels of detoxication enzymes and pointed toward some insensitive resistance mechanism.

Plapp and Casida (1969) reported that genes on autosome 2 and 5 in two house fly strains, controlled the tissue level of NADPH (reduced nicotinamide adenine dinucleotide phosphate). NADPH levels were controlled genetically to confer resistance to chlorinated hydrocarbon, pyrethroid, organophosphate, and methyl carbamate insecticides.

Plapp (1970) used back crosses of two carbamate resistant house fly strains to demonstrate resistance inheritance. By isolating heterozygotes with Isolan and carbaryl, Plapp distinguished resistant flies phenotypically and chemically. Isolated genes were located on chromosome 2; however, genes on chromosome 3 and 5 contributed insignificantly.

Resistance Mechanisms

The mechanisms of pesticide resistance are classified into two categories; behavioral and physiological. Behavioral resistance is defined as those actions that have evolved as the result of pesticide selection which aid the organism in avoiding toxicosis (Lockwood et al. 1984). Insects that are behaviorally resistant usually avoid pesticide residues and treated surfaces either by direct stimulation or host and/or habitat selection.

Physiological resistance is categorized as follows: I. Physical or restricted cuticular penetration, II. Increased enzymatic detoxication, and III. Altered site or reduced sensitivity of a physiological endogenous target (Busvine 1971; Devonshire 1973; Plapp 1976; Oppenoorth and Welling 1976; Oppenoorth 1984; Hodgson and Motoyama 1984).

Reduced Penetration

Early researchers investigating the rate at which insecticides penetrated the cuticle of various insects (Eldefrawi and Hoskins 1961; Plapp and Hoyer 1968b; Ku and Bishop 1967; Camp and Arthur 1967; Hanna and Atallah 1971; Ahmad et al. 1980) correlated that rate with the rate of internal metabolism. Resistance attributed to the rate of penetration produced a comparable rate of metabolism except in those species that were deemed highly resistant (Ku and Bishop 1967). Busvine (1971)

reported on work of other researchers who tried to explain resistance by this route. Several researchers measured cuticle thickness in R and S insect strains while others measured the protein and lipid content in the cuticle of R and S insect strains (Oppenoorth and Welling 1976). Patil and Guthrie (1979) altered the lipid composition of house fly cuticle by feeding artificial diets with and without DL-carnitine and 2-dimethylaminoethanol. House flies with abnormally high cuticular phospholipids did not always show a decrease in insecticide absorption. Strain and insecticide differences showed trends toward reduced penetration, thus, partially supporting the theory that a gene for penetration resistance can alter the cuticular composition to slow the rate of insecticide moving into organisms. Generally, the slower penetration rate allows the usually slow metabolic detoxication process to protect the organism from toxicosis.

Busvine (1971) also cited one case where excessive peritrophic membrane development accounted for the rapid excretion of DDT in a mosquito strain. Ariaratnam and Georghiou (1975) reported slight, but not statistically significant differences in rates of metabolism in R and S strains of Anopheles albimanus Wiedemann to carbaryl. They concluded that high resistance in this mosquito strain was yet unidentified but alluded to reduced penetration as the probable cause. DeVries and Georghiou (1981b) found decreased cuticular penetration as one of the resistance mechanisms in a permethrin selected strain of house fly. Devonshire (1973) showed that the gene for house fly penetration resistance was located on chromosome 3.

Sinchaisri et al. (1978) reported cuticular penetration as a possible mechanism of resistance in Leucania separata Walker to methyl

parathion, fenithrothion, diazinon, and phenthoate because each chemical showed variable rates of penetration. They concluded that penetrability can be influenced by solubility, lipophilicity and hydrophilicity of a compound, thus accounting for the variability in penetration rates in this insect strain. Oppenoorth and Welling (1976) also agreed that the effectiveness of the penetration gene is dependent on the nature of the insecticide and its avenue of administration.

Altered Site Insensitivity

Altered site insensitivity varies among organisms and between pesticides. Altered site insensitivity can take the form of

- Less sensitive AChE to inhibition by carbamate and OP Compounds (Oppenoorth 1984).
- Kdr (knockdown resistance), where the immediate immobility of an organism treated with DDT or pyrethroids does not occur. This phenomenon was first observed in the house fly (Oppenoorth and Welling 1976) and has subsequently been found in the cattle tick (Busvine 1971).
- Target site change. Evidence of HCH and dieldrin (cyclodienes) resistance in several mosquito, house fly and bed bug strains suggests target site change because no differences in metabolism or cuticular penetration was found between R and S strains (Oppenoorth 1965; Oppenoorth and Welling 1976).

The nervous system is an integral part of an organism thus making it a suitable target for alteration, inhibition or direct poisoning. The nervous system of both vertebrates and invertebrates is the most exploited target site for natural poisons and the majority of organic

insecticides (carbamates, organophosphates and chlorinated hydrocarbons) (Shankland 1976).

There is a multitude of papers describing the function of this chemically mediated cholinergic system. Also, recent reviews employing electrophysiological techniques for measuring electrical impulses and the effects of substrates on axonal sodium channels have been published (Shankland 1976; Edwards 1980; Laufer et al. 1984). In view of the above published works, description and operations of the nervous system will not be described here.

It is general knowledge that carbamate and organophosphorus insecticides exert their toxic action on the nervous system by inhibiting acetylcholinesterase (AChE) (Oppenoorth and Welling 1976; Hodgson and Motoyama 1984). Kinetic studies have shown that AChE of some R species is less sensitive to inhibition than their S counterparts (Hodgson and Motoyama 1984; Plapp 1976; Oppenoorth 1984) indicating an alteration or site change (Oppenoorth and Welling 1976; Busvine 1971).

Site changes or alterations can occur quantitatively or qualitatively, i.e., more sites of action or less sensitive sites (Oppenoorth 1984). Site alterations have only been found in AChE. The first evidence of altered AChE was found in the red spider mite, Tetranychus urticae Koch, by Smissaret in 1964 (Plapp 1976; Busvine 1971; Oppenoorth and Welling 1976; Oppenoorth 1984). Other mite strains showing altered AChE had slight changes in an imidazole residue relative to the serine hydroxyl necessary for acetylcholine hydrolysis (Plapp 1976).

Roulston et al. (1969) showed that the R-AChE of a Biarra strain of cattle tick, Boophilus microplus (Canestrini), was less sensitive to inhibition by organophosphate and carbamate insecticides than a was susceptible strain. R-AChE of the Biarra tick strain also showed 60% less activity toward acetylthiocholine than did the susceptible strain suggesting that their enzymes were different. Hama and Iwata (1971 and 1978) and Yamamoto et al. (1977) found that a strain of green rice leafhopper, Nephotettix cinctipes Uhler, was resistant to organophosphates and selected carbamates by insensitive AChE. Hama and Iwata concluded that insensitive AChE was controlled genetically by an incompletely dominant autosome.

Devonshire and Moores (1984) showed that differences in R-AChE from house flies were unusual in having a greater affinity for acetylthiocholine converse to previous works where R-AChE showed less affinity for ATC. They concluded that AChE should be partially protected from inhibitors by substrates present in the synapse, even if the enzyme was not also intrinsically insensitive to inhibition.

Biochemical differences in R and S AChE of the house fly was described by Devonshire (1975). The R and S enzymes showed no differences electrophoretically when applied on the surface of polyacrylamide gels with a surfactant. In the absence of the surfactant, R-AChE produced two distinct electrophoretic bands indicating heterogeny or isozymic forms but acted as one enzyme in vitro. R-AChE showed slower organophosphate inhibition than the S-AChE in this house fly strain.

Altered AChE has been predominantly found in mosquitoes, house flies, planthoppers, ticks and several mite strains (Voss 1980). Voss (1980) found that a related armyworm species, Spodoptera littoralis

Boisduval, was resistant by this mechanism. These findings indicate that lepidopterous larvae that are exposed to heavy selection pressures from various insecticidal classes possess the capability of altered AChE resistance.

Oppenoorth et al. (1977) found house fly R-AChE in combination with other metabolic detoxication mechanisms providing resistance to paraoxon and tetrachlorvinphos. DeVries and Georghiou (1981a, 1981b) found that decreased nerve sensitivity to permethrin combined with reduced cuticular penetration provided resistance in another house fly strain.

AChE inhibition and axonal sodium channel interference by pesticides can selectively produce organisms that are resistant. Also important are the new techniques available for determining effects on these insect systems by extrapolations from giant axons of crayfish or squids.

Increased Detoxication

A compound which is biologically active by virtue of interactions with biochemical systems such as enzymes and membranes will be vulnerable to attack by other enzymes in the same cells and tissues (Terriere 1982). "Attack" denotes metabolism of the compound. Metabolism generally results in detoxication and subsequent elimination of the metabolized compound from the organism's system. The original function of the MFO system is assumed to be that of metabolizing toxic allelochemicals (Dowd et al. 1983) and to a lesser extent, juvenile hormones (Yu and Terriere 1975) followed by juvenile hormone analogues (Yu and Terriere 1978). A typical metabolic scheme indicative of most lipophilic insecticides is shown in Figure 1. This scheme was derived from the many studies of insecticide metabolism in various organisms.

Figure 1. Metabolism of lipophilic foreign compounds.

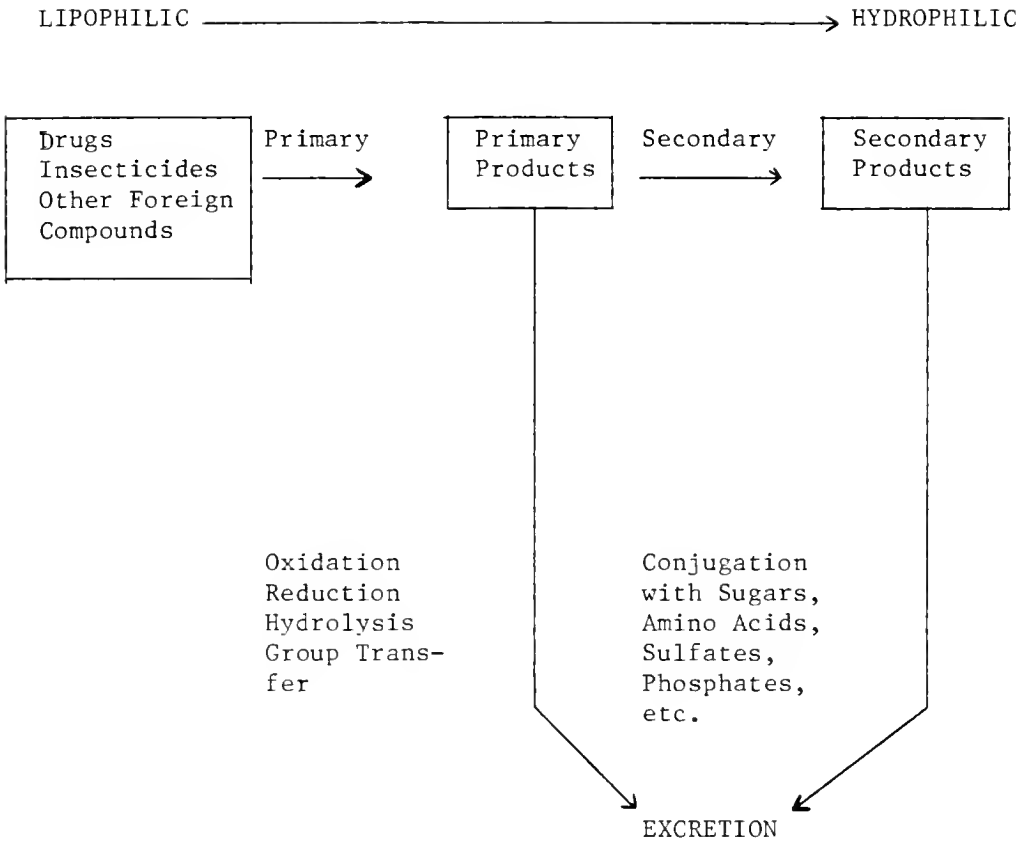


Figure 1. Metabolism of lipophilic foreign compounds.

Most of the more active insecticides are non-polar, lipophilic, fat soluble compounds which readily penetrate insect cuticle and gut walls. Non-polar compounds are usually insoluble in water; therefore, they are difficult to excrete without some biochemical modifications. However, some insects have developed the ability of rapidly excreting intact unchanged toxic molecules (Devonshire 1973; Matthews 1980; Ivie et al. 1983). Insects that possess this ability are considered highly resistant by virtue of rapid elimination.

Metabolism of lipophilic compounds may follow primary and/or secondary pathways, (Fig. 1) (Wilkinson and Brattsten 1972). Primary metabolism of lipophilic compounds takes the form of oxidation, reduction, group transfer, or hydrolysis. Some primary products are biotransformed into hydrophilic, water soluble products and are readily excreted. Those primary products that are not readily excretable are biotransformed into secondary products which are conjugated either with sugars, amino acids, phosphates, sulfates, glutathione or other endogenous conjugative compounds and excreted (Wilkinson 1983; Terriere 1982; Hollingworth 1976).

Cytochrome P-450 Mono-oxygenases

The most important oxidase enzymes are found in the endoplasmic reticulum membranes of cells. Cells which contain the most abundant oxidase enzymes are species specific. That is to say some organisms show higher oxidative activity from preparations of the midgut (Krieger and Wilkinson 1969; Yu and Ing 1984), fatbodies (Kuhr 1971; Price and Kuhr 1969; Brattsten et al. 1980), and less activity in preparations from malpighian tubules, fore- and hindgut, and the whole body (Krieger and Wilkinson 1969; Yu 1982b).

Fragmented endoplasmic reticulum membranes are called microsomes and are the results of tissue grinding or homogenation. The oxidase enzymes associated with microsomes are termed microsomal oxidases (Yu 1983a), mixed-function oxidases (MFO), or cytochrome P-450-dependent mono-oxygenases.

The MFO system accomplishes its functions by inserting one atom of molecular oxygen into a xenobiotic and combining the other oxygen atom with hydrogens from NADPH to form water (H_2O). Wilkinson (1983) depicted a generalized reaction for this procedure:

$$RH + O_2 + NADPH + H^+ \text{-------} > ROH + H_2O + NADP^+$$

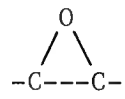
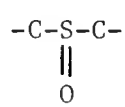
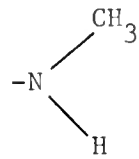
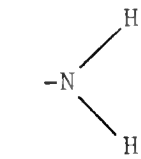
RH represents the lipophilic toxicant.
ROH represents the hydrophilic metabolite.

In the above reaction, electrons flow from NADPH + H^+ and a flavoprotein, cytochrome P-450 reductase (Terriere 1982) to an enzyme known as cytochrome P-450. Cytochrome P-450 binds to the xenobiotic (RH) and to oxygen (O_2) resulting in the splitting of molecular oxygen, inserting one atom in the xenobiotic (ROH) and combining the other with hydrogens from NADPH + H^+ to form water.

MFO actions on xenobiotics including insecticides are listed in Table 1, which was derived from Terriere (1982) and Yu (1982, personal communications).

The diversity of compounds attacked by MFO is shown to some extent in Table 1. The wide tissue distribution of MFO systems in insects demonstrates the ubiquitous nature of this important enzyme system. Increases in the rate of deactivation (detoxication) of toxic molecules can demonstrate the evolution of a resistance mechanism, particularly if

Table 1. The primary action of MFO systems on specific chemical configurations found in xenobiotic molecules.

Reaction Type	Chemical Configuration	Reaction Products	Consequence*
Epoxidation	-C=C-		Activation
Sulfoxidation	-C-S-C-		Activation
Phosphorothioate Oxidation			Activation
N-Dealkylation			Deactivation
O-Dealkylation	-O-CH_3	-C-OH	Deactivation
Hydroxylation	-C-H	-C-OH	Deactivation

* Activation means the metabolite is more toxic than the parent compound; deactivation means the metabolite is less toxic than the parent compound.

that rate is high enough to protect the organism from toxicosis. Indeed, this phenomenon occurs widely in the insect world. Insecticides detoxified by increased oxidation include DDT, carbamates, organophosphates and pyrethroids (Devonshire 1973).

Increased MFO deactivation of diazinon and diazoxon in a resistant house fly strain compared to a susceptible strain was demonstrated by Yang et al. (1971). Kuhr (1971) found increased fatbody MFO responsible for resistance in a cabbage looper strain to carbaryl. Feyereisen (1983) found high oxidative metabolism in a resistant house fly strain when measuring NADPH:cytochrome C reductase, cytochrome P-450 and aldrin and heptachlor epoxidase systems.

Multiple forms of cytochrome P-450 have been credited for the ability of insects to metabolize almost any foreign compound (Wilkinson 1983). Yu and Terriere (1979) found different forms of cytochrome P-450 in resistant and susceptible house fly strains. The resistant strain showed absorbance maxima lower than that found in the susceptible strain which resembled the high spin hemoprotein type cytochrome found in mammals. Terriere et al. (1975) used temperature, pH, ionic buffer strength and spectral data to determine microsomal oxidase differences in several R and S house fly strains. They found that a WHO standard reference strain showed abnormalities in the oxidase enzyme system and concluded that this strain may not be suitable as a reference strain. Also, this work suggested the presence of multiple forms of cytochrome P-450 as described by Yu and Terriere (1979).

Moldenke et al. (1984) isolated two forms of cytochrome P-450 from a house fly strain with different absorbance maxima and aldrin epoxidase activities. O-demethylase activity was detectable in one cytochrome P-450 fraction and not the other.

The MFO system is even more flexible in the metabolism of various chemical compounds. The induction of MFO systems provides this flexibility (Brattsten et al. 1977). Yu et al. (1979) and Berry et al. (1980) showed that peppermint plant leaves induced microsomal oxidases and cytochrome P-450 in the variegated cutworm. Brattsten et al. (1980) showed that epoxidation, N-demethylation and cytochrome P-450 reductase could be induced with phenobarbital either in midgut or fat body preparations from the southern armyworm. Yu and Ing (1984) demonstrated that another oxidase, fall armyworm microsomal hydroxylase, was induced by allelochemicals, drugs and host plants. Wood et al. (1981) and Farnsworth et al. (1981) showed that certain host plants could increase the tolerance in the fall armyworm and cabbage and alfalfa loopers when fed host plants that induced microsomal oxidases.

MFO induction appears to be age dependent. Yu (1982b, 1983a) showed that MFO in young fall armyworm larvae were less inducible than in older larvae. MFO induction also appears to be host plant and insect specific. Brattsten et al. (1984) showed that certain monoterpenes isolated from carrots induced MFO in the southern armyworm.

The significance of induction to the survival of an organism is yet unclear (Busvine 1971; Oppenoorth and Welling 1976; Wilkinson 1983); however, Perry et al. (1971) viewed chemical induction as a possible enhancement to the development of insect resistance.

Hydrolases

Hydrolases are those enzymes that catalyze the cleavage of molecules with water thus producing an acid and a leaving group, usually an alcohol or an amide. These include the esterases, phosphotases and amidases (Terriere 1982). Each group contains several different kinds

of hydrolases. Oppenoorth and Welling (1976) and Dauterman (1976) emphasized the importance of hydrolase attack on ester groups of many insecticides such as organophosphates, carbamates and pyrethroids but stated that the effects on organophosphates are most important in resistance.

A. Phosphotriester Hydrolysis

Phosphotriester hydrolase has been named DFP-ase, paraoxonase, A-esterase, phosphorylphosphatase, aryl esterase, phosphatase, etc. (Dauterman 1983). This enzyme or enzyme complex catalyzes the hydrolysis of organophosphate insecticides to produce phosphorus containing molecules that are poor cholinesterase inhibitors and are generally water soluble (Dauterman 1976, 1983).

B. Arylester Hydrolysis

Arylester hydrolases are implicated in the detoxication of aryl esters of organophosphorus compounds such as parathion or paraoxon (Dauterman 1976). Ahmad and Forgash (1976) described arylester hydrolases as 1) preferentially reacting with phenolic esters, 2) being inhibited by PCMB (parachloromercuribenzoate), 3) being activated by Ca_2+, and 4) readily hydrolyzing organophosphate compounds.

C. Carboxylester Hydrolysis

Carboxylesterases are known to catalyze the hydrolysis of aliphatic and aromatic carboxyl esters (Dauterman 1976; Ahmad and Forgash 1976) in many insecticides and is responsible for resistance. The hydrolysis of malathion by carboxylesterases produces malathion acid(s) and an alcohol(s). Zettler (1974) found that the carboxylesterase titre in a malathion resistant Indian meal moth strain was greater than in that of a susceptible strain. He also concluded that this strain of Indian meal

moth was resistant only to malathion and not other organophosphate compounds. Devonshire and Moores (1982) characterized carboxylesterase from the peach-potato aphid and found that the enzyme had broad substrate specificity thus contributing to organophosphate, carbamate and possibly pyrethroid resistance.

Motoyama et al. (1980) described a house fly strain that had multiple resistant mechanisms responsible for organophosphorus resistance. They concluded that a carboxylesterase from the nuclei, the mitochondria and the microsomal fraction was predominantly responsible for malathion resistance in this fly strain. Kao et al. (1984) selected two susceptible house fly strains with malathion and found that carboxylesterase activities and LD_{50} values were significantly increased after treating only three generations. Carboxylesterases were credited for rapid development of resistance to malathion in this house fly strain.

Hemingway and Georghiou (1984) found a mosquito strain resistant to organophosphorus insecticides by increased levels of esterase enzymes. They were able to reverse resistance below the susceptible level by treating the larvae with known esterase inhibitors, IBP (S-benzyl O,O-diisopropyl phosphorothioate), DEF (S,S,S-tributyl phosphorotrithioate) and TPP (triphenyl phosphate), thus partially confirming the resistance mechanism.

Recent studies of synthetic pyrethroid resistance have shown that hydrolases are responsible for flucythrinate, decamethrin, and fenvalerate resistance in an Egyptian cotton leafworm strain (Riskallah 1983). Resistance to another synthetic pyrethroid, permethrin, was found in a predatory mite strain by Scott et al. (1983). Several mite strains were investigated that had a prior exposure to DDT, azinphos-

methyl, carbaryl, and permethrin. In all cases, resistance was due to either a kdr type resistance or to increased ester hydrolysis. Hydrolase activity is generally measured with one of these commonly used substrates, α-naphthyl acetate (α-NA), β-naphthyl acetate (β-NA) and/or p-nitrophenyl acetate (p-NPA). Comparison of hydrolase activities of susceptible and resistant insects is a good measure of hydrolase resistance.

Glutathione S-transferases

Glutathione S-transferases are enzymes that catalyze the conjugation of glutathione (GSH) with many foreign compounds (Chasseaud 1973). Chasseaud (1973) and Dauterman (1983) explained the two main roles of GSH S-transferase as the conjugation of potentially harmful electrophiles with the nucleophile, GSH, thus protecting cell nucleophilic centers which occur in proteins and nucleic acids. Secondly, GSH provides an avenue for excretion of the potentially harmful electrophile through the formation of anionic, water-soluble products. GSH S-transferases catalyzes two type of reactions, the conjugations of GSH with epoxides and unsaturated compounds and the substitution of GSH with alkyl and aryl halides (Dauterman 1983).

There are many such transferases as described by Ahmad and Forgash (1976). These authors listed all known transferases requiring GSH in the metabolism of insecticides. GSH S-transferases act directly on the insecticide without the need for hydroxylation by MFO.

Usui and Fukami (1977) found two transferases from cockroach fat bodies active on diazinon and three transferases active on methyl parathion. Wool et al. (1982) correlated high GSH S-transferase levels with resistance to malathion in a flour beetle strain. Motoyama et al. (1980) determined that resistance in a house fly strain was in part due

to elevated levels of GSH S-transferase. Oppenoorth et al. (1977) found GSH S-transferase levels in a resistant house fly strain 9 to 120-fold more than a susceptible strain to methyl parathion, parathion, methyl paraoxon and paraoxon.

GSH S-transferases are known to be induced by allelochemicals (Yu 1982a) and insecticides. Xanthotoxin, an allelochemical from parsnip, induced GSH S-transferase by 39-fold in an insecticide resistant and susceptible strain of fall armyworm (Yu 1984). Permethrin, a synthetic pyrethroid, induced GSH S-transferase 296% of the control when fed to groups of adult honey bees for two days (Yu et al. 1984). Hayaoka and Dauterman (1982) induced GSH S-transferases in a strain of house fly with phenobarbital and several chlorinated hydrocarbon insecticides. House fly pretreatment with phenobarbital afforded some protection from toxicosis by several organophosphorus insecticides, thus further emphasizing the importance of GSH S-transferases in insecticide detoxication.

Epoxide Hydrolases

Epoxide hydrolases are enzymes that hydrate epoxides of certain arene, alkene and cyclodiene compounds to trans-diols by the inclusion of water in the molecules (Dauterman 1976; Oesch et al. 1971). Enzymatic hydration of epoxides is recognized as an important metabolic reaction in protecting organisms from potentially hazardous labile epoxides which are considered carcinogens (Yu 1982, personal communication; Dauterman 1976). I have not found literature articles where epoxide hydrases contribute significantly to insect resistance; however, their presence is unquestionably important in the detoxication of cyclodienes such as dieldrin, enzymatically altered compounds such as heptachlor epoxide, and other more stable deleterious epoxides.

History of Carbaryl Resistance

Carbaryl was introduced to the commercial market in 1956 to control a variety of insect pest species including those that were highly resistant to DDT (Harding and Dyar 1970). The first reported cases of resistance to this compound were against the light brown apple moth in 1963 in New Zealand and in 1966 against the tobacco budworm in the U.S. (Mount and Oehme 1981).

Since carbaryl controlled important agronomic and urban insects, it was no surprise when Ku and Bishop (1967) reported that carbaryl resistance in a cockroach strain was due to three resistance mechanisms. The primary mechanism was reduced cuticular penetration while increased excretion and metabolism contributed significantly to the elevation of resistance in this strain.

Roulston et al. (1968, 1969) reported insensitive AChE in a Biarra strain of cattle tick while Schuntner et al. (1972) found increased metabolism responsible for resistance in a Mackay strain of cattle tick.

Increased oxidative metabolism was found to be responsible for carbaryl resistance in a resistant cabbage looper strain (Kuhr 1971). Atallah (1971) selected several strains of Egyptian cotton leafworms with carbaryl for 15 generations and found a 30-fold increase in resistance. Biochemical identification of the resistance mechanism proved to be increased metabolism and restricted cuticular penetration (Hanna and Atallah 1971). Atallah (1971) simultaneously selected individuals of the same leafworm strain used for carbaryl selection with DDT. He found that DDT resistance developed much more slowly than that of carbaryl. DDT resistance was 24-fold after 26 generations. This work indicated multifactorial resistance to carbaryl while DDT resistance was probably due to a single mechanism.

Hama and Iwata (1971) found insensitive AChE responsible for carbamate resistance, including carbaryl, in a resistant strain of green rice leafhopper. Hama and Iwata (1978) described the heritability of resistance in this leafhopper strain as being controlled by an incompletely dominant autosomal gene.

Wolfenbarger et al. (1981) reported increases in carbaryl LD_{50} values from the American bollworm, Heliothis armigera, from 1969 to 1973 in Thailand as 94 ug/g to 310 ug/g, respectively. Increases of this magnitude over this period indicate the tremendous insecticidal selection pressure applied to this insect.

Rose and Brindley (1985) showed that a carbaryl resistant Colorado potato beetle strain from New Jersey was resistant due to an increase in oxidative metabolism. Potato beetles in the northeastern U.S. are subjected to tremendous insecticide selection pressures because they have developed resistance to many of the highly toxic persistent insecticides including chlorinated hydrocarbons, organophosphates, and carbamates.

Fall Armyworm Resistance to Carbaryl

The fall armyworm is a highly mobile phytophagous pest of many grasses, corn, oats, rye, cotton, garden vegetables, and other succulent plants (Quaintance 1897). This species migrates from the tropics, Florida and Gulf coast states (Luginbill 1928; Vickery 1929) as far north as Canada (Snow and Copeland 1969; Combs and Valerio 1980). In 1979, using diet spray bioassay techniques, Young (1979) found that the fall armyworm was resistant to carbaryl. Many researchers believe that wide-spread resistance in this species could prove devastating to farmers from the tropics to Canada and west to southern California.

MATERIALS AND METHODS

Insects

R Strain

The carbaryl resistant strain of fall armyworm was collected near Tifton, Georgia, by Dr. J. R. Young. Larvae were reared on a meridic diet (Burton 1969). Environmental conditions were 27 ± 2 degrees C with 50 ± 5% relative humidity and 16:8 light:dark photoperiod. Moths were held in a separate environmental chamber whose atmospheric conditions were 26 ± 2 degrees C, 50 - 70% relative humidity, and 16:8 light:dark photoperiod.

S Strain

Eggs of the carbaryl susceptible strain were obtained twice weekly from the United States Department of Agriculture (USDA), Gainesville, Florida. Larvae were reared under the same conditions as the R strain; however, old sixth instar larvae were discarded.

The rearing procedures utilized have been previously described by Young (personal communication) and Shorey and Hale (1965). Modifications to each were made to accommodate current laboratory conditions. Moths of the R strain were housed in one-gallon cardboard ice-cream containers. The lid was removed and fitted with an absorbent paper towel. The moths were fed a 10% sucrose solution saturated on sterile cotton in a 4 oz squat cup. Eggs were removed thrice weekly by anesthetizing the moths with 12 second bursts of CO_2. The moths were

transferred to a clean container and provided clean towelling and fresh sucrose solution (10%).

Eggs on paper towelling were sterilized in a 10% formaldehyde solution, rinsed in tap water and allowed to dry. The paper towelling was glued to tab lids of 16 oz plastic cups, each containing about ½ inch of the artificial diet.

Chemicals

[^{14}C] carbaryl was purchased from the California Bionuclear Corporation, Sun Valley, CA, and [8-^{14}C] styrene oxide was purchased from the Amersham Corporation. All insecticides and chemical reagents were of the highest purity available commercially. Carbaryl metabolites were a gift from The Union Carbide Corporation.

Bioassay

The bioassay methods used were as described by Mullins and Pieters (1982). Twenty 4th instar Spodoptera frugiperda larvae (22 ± 3 mg in weight) were placed into a four inch glass petri dish. An ISCO Model M microapplicator was used to treat the larvae topically on the dorsal prothorax with 1 ul of insecticide diluted with acetone. Controls were treated with 1 ul of acetone only. After treatment, the larvae were transferred individually to glass scintillation vials, each containing about 1 gram of artificial diet. Mortality was recorded 24 and 48 hours post-treatment with the end point being a completely moribund condition unresponsive to prodding. Only 48 hour data were used in probit analysis. All insecticides were tested at a minimum of five dosages, on at least four different days. Probit analyses were made by a computer program.

Protein Determinations

The protein content of each preparation, midgut microsomal suspension or crude homogenate was measured by the method of Bradford (1976).

A protein reagent was made by adding precisely the ingredients described by Bradford:

a). Coomassie Brilliant Blue G-250 dye (100 mg)

b). Ethanol-95% (50 ml)

c). Phosphoric acid-85% (100 ml)

This solution was brought to a final volume of 1 liter, stirred, filtered twice and used for all assays. A standard curve was made with multiple determinations of known quantities of bovine serum albumin (BSA), Fraction V.

A typical mixture included 0.1 ml of 10 ug BSA protein pipetted into a test tube and 3.0 ml protein reagent added. This mixture was shaken and incubated at room temperature for a minimum of 2 minutes. A blank was prepared with 0.1 ml warm 0.1 M sodium phosphate buffer, pH 7.5, plus 3.0 ml protein reagent and handled as above.

A desk top Turner Model (330) single beam spectrophotometer was used to measure optical densities (O.D.) at 595 nm. Each protein concentration was replicated 3 times and run on at least 3 different days. The average O.D. was plotted on graph paper against micrograms of BSA protein to establish the standard curve.

To determine unknown protein quantities, 0.03 ml protein solution and 0.07 ml of 0.1 M sodium phosphate buffer, pH 7.5, were added to a standard test tube. A volume of 3.0 ml protein reagent was added, and the tube shaken and incubated at room temperature for a minimum of 2 minutes. Optical densities were measured at 595 nm and compared to the standard curve.

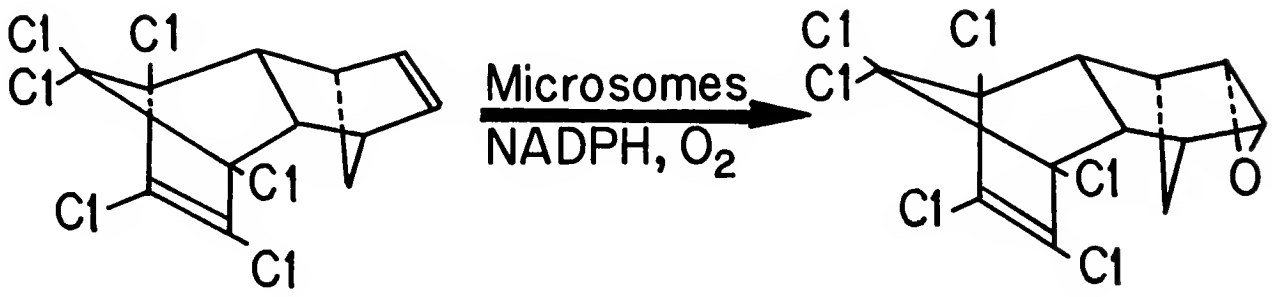
Epoxidation Assay

Aldrin epoxidation was assayed (Fig. 2) by the method of Yu et al. (1979) and Yu and Terriere (1979). Aldrin epoxidation was assayed with

two types of enzyme preparations, crude homogenate and microsomal fraction. Crude homogenates were uncentrifuged homogenates of fall armyworm midguts. They were obtained by dissecting larval midguts, removing the food containing peritrophic membrane and placing the cleaned guts into ice-cold 1.15% KCl solution. The clean guts were then transferred to an ice-cold glass homogenizer tube into which 20.0 ml ice-cold 0.1 M sodium phosphate buffer, pH 7.5, were added. The guts were homogenized for about 30 seconds with a motor-driven teflon tissue grinder. Homogenized guts were filtered through double layer cheesecloth and used as the enzyme source. Microsomal isolation followed the above steps except the homogenate was centrifuged in a Beckman L5-50E ultracentrifuge at 10,000g max at 0 to 4 degrees C for 15 minutes. The pellet containing mitochondria and cell debris was discarded and the supernatant filtered through glass wool. The supernatant was recentrifuged at 105,000g max for 65 minutes. The resulting microsomal pellet was resuspended in ice-cold 0.1 M sodium phosphate buffer, pH 7.5, to obtain a protein concentration 1.0 mg/ml and used immediately as the enzyme source. A typical 5 ml incubation mixture contained 0.1 M sodium phosphate buffer, pH 7.5, an NADPH generating system (1.8 umoles of NADP; 18 umoles of glucose-6-phosphate; 1.0 unit of glucose-6-phosphate dehydrogenase); 250 nmoles of aldrin in 0.1 ml methyl Cellosolve; and 2.0 ml of microsomal suspension (1 mg protein). Mixtures were incubated in a water bath while being shaken at 30 degrees C in an atmosphere of air for 15 minutes. Each incubation was duplicated and accompanied by a blank or control which did not contain microsomes. After 15 minutes, each reaction was stopped by adding 10 ml hexane and placing the incubation tube on ice. The epoxidation product, dieldrin, was extracted

Fig. 2. The reaction of aldrin with midgut microsomes to produce the epoxide product, dieldrin.

Aldrin epoxidase



Aldrin

Dieldrin

from the mixture by slowly shaking it for one hour. Dieldrin formation was analyzed on a Varian Model 3740 gas chromatograph equipped with an electron capture detector. The column was 4 ft. X 2 mm i.d. glass, packed with a 1:1 mixture of 5% DC 11 and 5% QF 1 on 100 to 120 mesh high performance Chromosorb W (Yu and Terriere 1974; Yu 1982).

Microsomal Biphenyl Hydroxylase Assay

Microsomal biphenyl hydroxylation (Fig. 3) is a mixed-function oxidase system that plays a major role in the oxidative metabolism of foreign substances in insects (Yu and Ing 1984). The activity of this enzyme system in the fall armyworm was determined by the method of Yu and Ing (1984) which used biphenyl as substrate.

Microsomes were isolated from 25 cleaned guts by homogenizing the guts in 20 ml ice-cold 0.1 M sodium phosphate buffer, pH 7.5, and centrifuging as above. The resulting microsomal pellet was resuspended in ice-cold 0.1 M sodium phosphate buffer, pH 7.5, and used immediately as the enzyme source. A typical 5.0 ml incubation mixture contained 0.3 ml of an NADPH generating system as mentioned above; 2.6 ml of a 0.1 M sodium phosphate buffer, pH 7.5; 2.5 mg biphenyl in 0.1 ml methyl Cellosolve, and 2.0 ml of microsomal suspension (1 mg protein). Mixtures were incubated in duplicate in a water bath while being shaken at 30 degrees C in an atmosphere of air for 30 minutes.

The reactions were stopped by adding 5.0 ml ethyl acetate and placing incubation tubes on ice. The hydroxylated product, 4-hydroxybiphenyl, was extracted twice with 5.0 ml of ethyl acetate, each time, dried over anhydrous sodium sulfate and analyzed by high performance liquid chromatography (HPLC). Analyses were performed on a Beckman Series 340 HPLC at 254 nm. The column was an Ultrasphere-Si measuring

Fig. 3. The reaction of biphenyl with microsomes to produce the oxidative metabolite 4-hydroxybiphenyl.

Biphenyl 4-hydroxylase

Biphenyl

4-hydroxybiphenyl

25 cm X 4.6 mm i.d. Isopropanol (5%) in hexane was used to elute the column at a flow rate of 0.75 ml/minutes. Enzymes were denatured by heat and tested as above to determine non-enzymatic product formation.

Microsomal N-Demethylase Assay

Microsomal N-demethylation of p-Chloro-N-methylaniline (PCMA) (Fig. 4) was carried out by the method of Kupfer and Bruggeman (1966). Standard curves were obtained by measuring, spectrophotometrically, known concentrations of p-Chloroaniline (PCA) in an aqueous solution at 445 nm. All assays, whether standard curve determinations or enzyme activity determinations, consisted of a comparable blank, i.e., the absence of PCA or the use of heat denatured protein. A Beckman Model 5260 spectrophotometer was used for all N-demethylation assays.

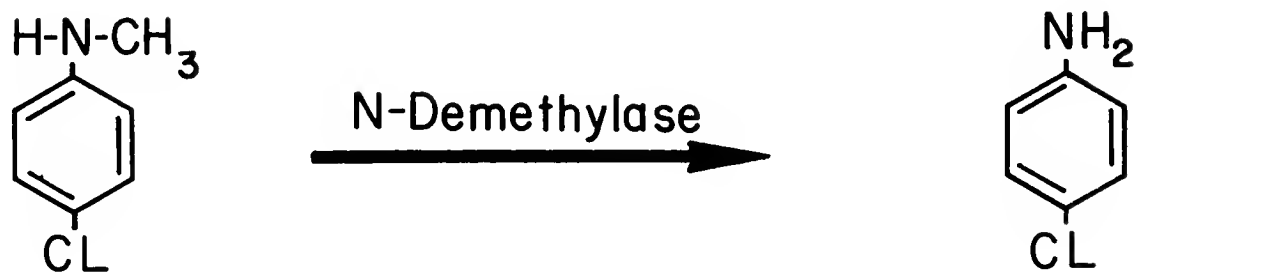
Microsomes were prepared as mentioned earlier and suspended in 0.1 M sodium phosphate buffer, pH 7.5. A 5.0 ml incubation mixture contained 0.3 ml NADPH generating system (1.8 umoles of NADP; 18 umoles of glucose-6-phosphate, and 0.5 unit of glucose-6-phosphate dehydrogenase); 0.1 ml PCMA (30 umoles in aqueous HCl); 2.6 ml of 0.1 M sodium phosphate buffer, pH 7.5; and 2.0 ml microsomal preparation (0.5 to 1 mg protein/ml). The incubation mixture was shaken at 34 degrees C for 20 minutes. The reaction was stopped with 2.0 ml of a 6% aqueous p-dimethylaminobenzldehyde and centrifuged for 15 minutes at 10,000 RPM in a refrigerated Beckman Model JA-21 centrifuge. The incubation tubes were allowed to reach ambient temperature before being analyzed spectrophotometrically at 445 nm on a Beckman Model 5260 spectrophotometer. Each incubation was duplicated and each experiment was repeated three times.

Cytochrome P-450 Measurement

Cytochrome P-450, a carbon monoxide-binding pigment of endoplasmic reticulum, was determined by the method of Omura and Sato (1964).

Fig. 4. The reaction of p-Chloro-N-methyl aniline with microsomes to produce the demethylated product p-Chloroaniline.

Microsomal N-Dealkylation



p-Chloro-N-methyl Aniline

p-Chloro Aniline

Midgut microsomes from 20 cleaned guts were homogenized in 20 ml of ice-cold 0.1 M sodium phosphate buffer, pH 7.5, and centrifuged as above. The resulting microsomal pellet was resuspended in ice-cold 0.07 M sodium phosphate buffer, pH 7.5, containing 30% glycerol and used immediately as the enzyme source.

Baseline scans of the microsomal suspension alone were run on a Beckman Model 5260 uv/vis spectrophotometer equipped with a scattered transmission accessory at 300 to 500 nm. After recording the baseline, the sample cuvette was removed and carbon monoxide (CO) was gently bubbled through the preparation for 1 minute. This sample was reduced with a few milligrams of sodium dithionite ($Na_2S_2O_4$), stirred with a glass rod and again scanned from 300 to 500 nm. Scanning was continued until a maximum spectrum was obtained. This assay was duplicated and run on at least 3 different days on both insect strains (Yu 1982b).

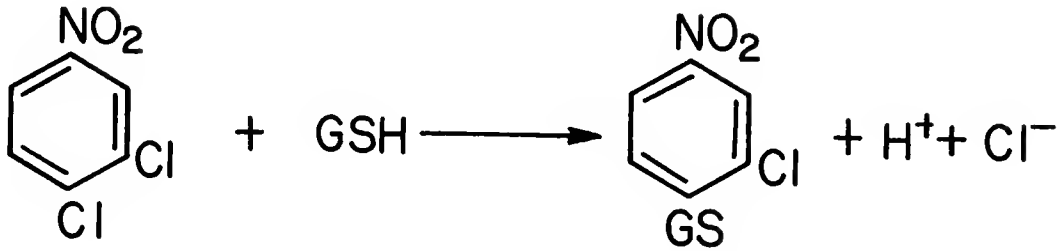
Glutathione S-Transferase Assay

Glutathione S-transferases (Fig. 5) are enzymes that catalyze the conjugation of glutathione (GSH) with many foreign compounds (Chausseaud 1973). Conjugation products are usually water soluble, readily excretable substances and their formation generally results in a decrease in xenobiotic toxicity (Yang 1976).

Glutathione S-transferase activity was measured by the method of Yu (1982a). Midgut soluble enzyme fractions were used in lieu of the resuspended microsomal pellet. Twenty cleaned guts were homogenized in 20 ml of ice-cold 0.1 M Tris-HCl buffer, pH 9.0, and filtered through double layered cheesecloth. The homogenate was centrifuged at 10,000g max for 15 minutes. The resulting supernatant was filtered through glass wool and recentrifuged at 105,000g max for 65 minutes. Prior to decanting the supernatant from the centrifuge tube, all lipids were

Fig. 5. The reaction of 3,4-dichloronitrobenzene with glutathione by the enzyme Glutathione S-aryltransferase to produce the conjugated product S-(2-chloro-4-nitrophenyl) glutathione.

Glutathione S-aryltransferase



3,4-dichloro-nitrobenzene

S-(2-chloro-4-nitrophenyl) glutathione

removed from the supernatant surface with a medicine dropper and discarded. The supernatant was then gently poured into a large test tube so as not to disturb the microsomal pellet and kept on ice for immediate use. A typical 3.0 ml reaction mixture contained 1.0 ml of 15 mM glutathione and 2.0 ml soluble fraction (2.0 ml 0.1 M Tris-HCl buffer, pH 9.0, served as blank) was first incubated for 3 minutes at 37 degrees C, after which 0.02 ml 150 mM 1,2-dichloro-4-nitrobenzene (DCNB) was added and mixed. The change in absorbance at 340 nm for 5.0 minutes was measured with a Beckman Model 5260 uv/vis spectrophotometer. The enzyme activity was expressed as nmoles DCNB conjugated per minute per milligram of protein using an extinction coefficient of 10 mM^{-1} cm^{-1} for S-(2-chloro-4-nitrophenyl) glutathione.

In vitro Carbaryl Metabolism Study

Carbaryl was metabolized *in vitro* by modifications of the methods of Kuhr and Davis (1975), Kuhr and Hessney (1977), and Yu and Terriere (1978). Midgut homogenate was prepared, as described earlier, from 2 day-old sixth instar larvae to obtain 4-5 mg protein/ml.

Midgut homogenates of R and S larvae were incubated with carbaryl in an atmosphere of air for 2 hours at 30 degrees C. The 5.0 ml incubation mixture contained 0.3 ml of an NADPH generating system, as mentioned above; 0.58 ml of 0.1 M sodium phosphate buffer, pH 7.5; 0.437 ug [^{14}C] carbaryl (100,000 dpm); 10 ug cold carbaryl; 10 mg bovine serum albumin; 0.05 ml methyl Cellosolve; and 4.0 ml of midgut crude homogenates. The NADPH generating system was omitted from some incubations in order to study nonoxidative metabolism of carbaryl. The incubation mixture was stopped with 5.0 ml chloroform, and carbaryl and its metabolites were extracted by the solvent. The same extraction was repeated again and the combined extracts were then dried over anhydrous sodium

sulfate. Two milliliter aliquots of chloroform were concentrated under a stream of air to 0.2 ml and spotted on silica gel G thin layer chromotographic (TLC) plates (0.25 mm). The TLC plates were developed in a solution of acetic acid:ethyl acetate:benzene (1:10:33 by volume) and scanned for radioactivity in a Packard Model 7220/21 radiochromatogram scanner. Individual spots were identified by R_fs of previously chromatographed standard metabolites (see Table 2). Each peak was scraped from the plates and counted in a Tracor Analytic Data 300 liquid scintillation counter. The oxidative metabolites were combined due to poor separation and resolution near the TLC plates' origin.

Epoxide Hydrolase Assay

Epoxide hydrolase (Fig. 6) was assayed by the method of Yu et al. (1984) using [^{14}C] styrene oxide as substrate. Microsomes were prepared as previously described and suspended in 0.5 M Tris-HCl buffer, pH 9.0, to make a final concentration of 0.4 mg protein/ml. Heat denatured enzyme was used as the control to correct for any non-enzymatic glycol formation.

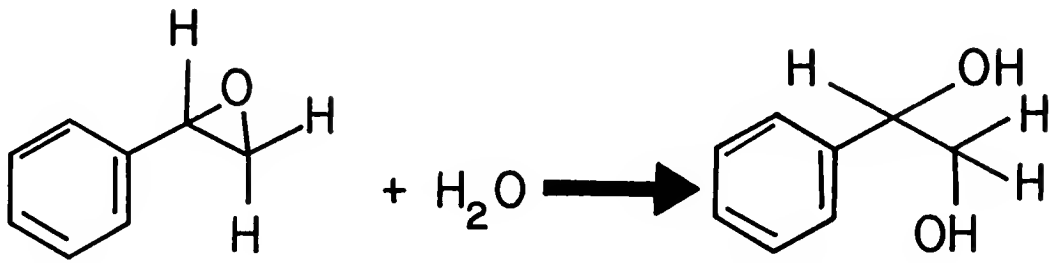
Screw cap tubes were used to hold the incubation mixture which contained 0.6 ug (100,000 dpm) [^{14}C] styrene oxide, 8.0 ug cold styrene oxide in 7.0 ul of acetonitrile, and 0.5 ml microsomal suspension. This mixture was incubated in a shaking water bath at 37 degrees C for 5 minutes. The reactions were stopped by the addition of 10 ml petroleum ether and the unreacted styrene oxide was extracted by the solvent. The petroleum ether was readily decanted by freezing the aqueous phase in a dry ice-acetone mixture. The same extraction was repeated again after the aqueous phase was thawed. The aqueous solution which contained the polar product, [8-^{14}C] styrene glycol, was then shaken with 2 ml ethyl

TABLE 2. R_f values of carbaryl and its metabolites on silica gel G plates in a developmental solution of acetic acid:ethyl acetate:benzene (1:10:33 by volume).

Compound	R_f
α-naphthol	0.79
Carbaryl	0.64
5-hydroxy-carbaryl	0.49
4-hydroxy-carbaryl	0.40
Methylol-carbaryl (N-hydroxymethyl)	0.29

Fig. 6. The reaction of [^{14}C] styrene oxide with water and microsomes to produce the water soluble product, styrene glycol.

Epoxide hydrolase



Styrene oxide

Styrene glycol

acetate, and the product in the ethyl acetate was quantified by liquid scintillation counting.

Esterase Assays

Esterases were assayed by the method of van Asperen (1962) (Fig. 7) using α-naphthylacetate (α-NA) as substrate. Both midgut microsomes and crude homogenates were used to perform this assay. A typical 6.0 ml incubation mixture contained 4.95 ml of 0.04 M sodium phosphate buffer, pH 7.0; 0.05 ml of a 0.03 M α-NA in acetone, and 1.0 ml of midgut homogenate or microsomal preparation. To assay for carboxylesterase activity, eserine (10^{-4} M) and p-hydroxymercuribenzoate (PHMB) (10^{-4} M) were added to the incubation mixture to inhibit cholinesterase and arylesterases, respectively.

This mixture was incubated for 30 minutes at 27 degrees C and the reaction was stopped by placing each incubation tube on ice and introducing 1.0 ml of diazoblue laurylsulfate solution (DBLS). A red color developed and quickly changed to a dark blue color. The absorbance of the reaction product, naphthol-diazoblue, was measured at 600 nm on a Beckman Model 5260 spectrophotometer against a blank containing no enzyme. Optical densities of the reaction products were compared to known quantities of naphthol reacted with DBLS and plotted as a standard curve.

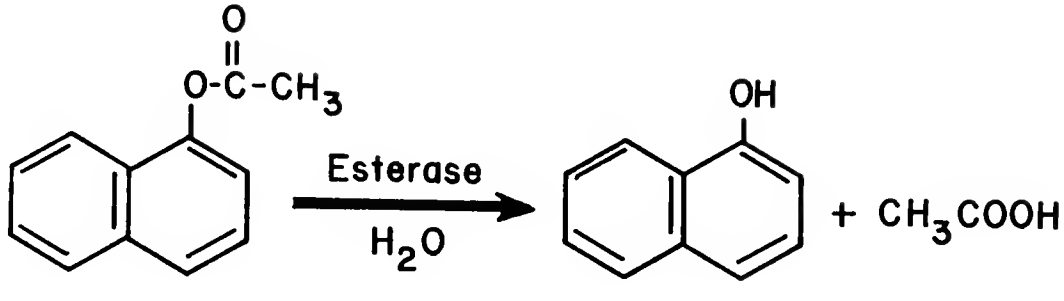
All incubations were duplicated and each experiment was repeated twice.

Acetylcholinesterase Assay

Acetylcholinesterase (AChE) (Fig. 8) was assayed by the method of Ellman et al. (1961) using acetylthiocholine (ATC) as substrate. Initially fall armyworm adult heads, whole larvae and larval heads were

Fig. 7. The reaction of α-naphthylacetate with esterases to form α-naphthol and acetic acid.

Esterases



α-naphthylacetate

α-naphthol

Fig. 8. The reaction of acetylthiocholine with acetylcholinesterase producing thiocholine which produced a yellow color when combined, in reaction, with 5-dithiobis-2-nitrobenzoic acid.

Acetylcholinesterase

1. $(CH_3)_3{}^+NCH_2CH_2S-\overset{O}{\overset{\|}{C}}CH_3 + H_2O \xrightarrow{AchE} (CH_3)_3{}^+NCH_2CH_2S^- + CH_3COO^- + 2H^+$

   acetylthiocholine — thiocholine

2. $(CH_3)_3{}^+NCH_2CH_2S^- + O_2N\text{-}C_6H_3(COO^-)\text{-}S\text{-}S\text{-}C_6H_3(COO^-)\text{-}NO_2 \longrightarrow$

   thiocholine — 5-dithiobis-2-nitrobenzoic acid

$(CH_3)_3{}^+NCH_2CH_2S\text{-}S\text{-}C_6H_3(COO^-)\text{-}NO_2 + O_2N\text{-}C_6H_3(COO^-)\text{-}S^-$

5-thio-2-nitrobenzoic acid

assayed for AChE activity. Whole larvae larval heads showed low activity and were not used. Adult heads contained the highest activity and were subsequently used in this assay.

Two-day old adults of both R and S strains were frozen and their heads removed with forceps. The heads were homogenized for 30 seconds in ice-cold 0.1 M sodium phosphate buffer, pH 8.0, in a glass homogenizer with a teflon pestle attached to a motorized grinder, for 30 seconds. The homogenate was filtered through doubled layered cheesecloth and used as the enzyme source.

A typical 3.2 ml incubation mixture contained 2.75 ml of 0.1 M sodium phosphate buffer, pH 8.0; 0.1 ml of 0.01 M 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 0.1 ml ice-cold 0.075 M acetylthiocholine (ATC); and 0.2 ml enzyme. The reaction was initiated by adding 0.2 ml enzyme to the incubation mixture. The blank contained all of the above reagents excluding the enzyme. The buffer was increased in the blank to compensate for the lack of enzyme. The yellow colored reaction product formation was measured for 5 minutes at 412 nm against the blank which showed some non-enzymatic ATC hydrolysis.

Cuticular Penetration by Carbaryl

Cuticular penetration of carbaryl was measured by a method modified from Ku and Bishop (1967). The cuticular penetration was assayed by topically applying [^{14}C] carbaryl to the dorso-prothorax of fall armyworm larvae. The treated larvae were rinsed with acetone at different time intervals after treatment. The excreta was extracted directly from scintillation vials with Scinti-Verse I scintillation cocktail. Carbaryl that penetrated larval cuticle was extracted by homogenizing whole larvae at various time intervals and washing the remaining carcasses and

utensils with aliquots of acetone. Washes were collected, concentrated, and radioactivity measured.

In a typical experiment, duplicate groups of 4 larvae each of 2-day-old sixth instar were placed into a glass petri dish. Each larva was treated topically with 1.0 ul acetone containing 0.048 ug [^{14}C] carbaryl and 0.952 ug cold carbaryl. Those larvae that were assayed at zero hour were rinsed immediately in three 5.0 ml aliquots of acetone in 3 separate scintillation vials. Those larvae requiring rinses at other time intervals were treated and placed into separate pre-labelled vials. Larvae were removed individually from each duplicate vial set at the designated time interval and rinsed as above. After being rinsed, each larva was cut into four pieces and placed into a glass homogenizer. After all larvae had been rinsed, the larval pieces (two larval equivalents) were homogenized in 7.0 ml acetone. The pestle was rinsed with another 5.0 ml acetone and the acetone was filtered into a 250 ml Erlenmeyer flask. The filter paper (Whatman #1) and the precipitate were rinsed twice into the same flask. The combined extracts were concentrated to approximately 5.0 ml under a constant stream of air. The Erlenmeyer flasks were rinsed 2 times with acetone, and the combined rinses were concentrated and added to the original extract in the scintillation vial.

Ten milliliters of Scinti-Verse I scintillation cocktail were added and radioactivity was counted in a Tracor Analytic Data 300 liquid scintillation counter.

Statistics

Computerized t- and F-tests were used to compute the significance of the difference between means and to determine population normality, respectively. All *in vitro* data, except carbaryl metabolism, were analyzed by these methods.

RESULTS

Bioassays

All FAW bioassays were by the methods of Mullins and Pieters (1982). Carbaryl, parathion, methomyl, diazinon, cypermethrin, and permethrin were bioassayed initially to determine FAW susceptibility to different insecticidal classes and to determine cross-resistance if any. The LD_{50} values (ug/g larva) for each insecticide are shown in Table 3 along with the relative resistance ratios in descending order of susceptibility. The resistant strain showed > 90X resistance to carbaryl but remained susceptible to a related carbamate, methomyl (4.7X). The resistant strain also showed tolerance to the organophosphates, parathion (5.8X), and diazinon (2.9X). The R and S fall armyworm strains showed no tolerance or cross-resistance to the synthetic pyrethroids, cypermethrin and permethrin.

In Table 4, the toxicological responses are shown for larvae treated with PB, a known microsomal oxidase inhibitor (Yu, 1982b). Resistance was reduced to 6X in the R strain when PB was topically applied in conjunction with carbaryl at a 5:1 ratio. These results suggest that microsomal oxidases are involved in carbaryl resistance. The synergized resistance level of the R strain did not approach that of the susceptible strain, further suggesting that other factors are possibly involved in carbaryl resistance. Also, the LD_{50} of the susceptible strain was reduced by PB, suggesting that microsomal oxidases play an important role in the detoxication of carbaryl in this insect strain.

TABLE 3. Comparison of toxicological responses of R and S fall armyworm larvae topically treated with 6 insecticides.

Insecticide	LD_{50} (ug/g Larva)[a/]		
	R	S	R/S
Carbaryl	10343[b/]	115	90
Parathion	14.38	2.46	5.85
Methomyl	4.17	0.98	4.74
Diazinon	19.18	6.50	2.95
Cypermethrin	0.12	0.08	1.50
Permethrin	0.23	0.17	1.35

a/ Each observation consisted of at least two different tests.
b/ Computer estimate

TABLE 4. Comparison of toxicological responses of R and S fall armyworm larvae topically treated with carbaryl + PB.

Insecticide /Synergist	LD_{50} (ug/g Larva)[a/]		
	R	S	R/S
Carbaryl	10343[b/]	115	90
Carbaryl + Piperonyl Butoxide	400.43	67	5.98

a/ Each observation consisted of at least two different tests.
b/ Computer estimate

These *in vivo* results may prove helpful in the discussion of results found in *in vitro* detoxication assays. DEF, (S,S,S,tributyl phosphorotrithioate), a known esterase inhibitor, was too toxic when applied alone for esterase comparisons. TOCP (tri-O-creosyl phosphate), another known esterase inhibitor, had no effect on the LD_{50} levels in either strain. These findings indicate that esterases do not play a role in carbaryl resistance in this FAW strain.

Enzymatic Assays

A. Aldrin Epoxidase

The specific activities of aldrin epoxidase enzyme of various larval instars are shown in Table 5. Larvae younger than fourth-instar were not studied because of the difficulty in dissecting the midgut. The R strain showed significantly higher epoxidase activity than the S strain across all instars tested. These results support those of Yu (1984) in that the R strain of fall armyworm possesses a higher level of microsomal epoxidase activity than does the S strain.

B. Biphenyl Hydroxylase

The results summarized in Table 6 show that the biphenyl 4-hydroxylase activity was significantly higher in R larvae. Larvae younger than 4th instar were not examined because of the difficulty in dissecting midguts. The activity of biphenyl 4-hydroxylase enzyme increased in both strains from 4th to 2 day-old sixth instar larvae. Three day-old sixth instar individuals showed a slight decrease in activity thus confirming results from Yu (1984) that the activity of important detoxication enzymes reached maximum capacity in the second day of the final instar. High activity begins to decline with the onset of pupation. Four day old sixth instar larvae were not observed feeding, and were

TABLE 5. Aldrin epoxidase activities of midgut microsomes and homogenates from various instars of R and S fall armyworm larvae.[a/]

Larval Instar	Specific Activity (pmol dieldrin min^{-1} $mg\ protein^{-1}$)	
	R	S
	Microsomes	
5th[b/]	483.23 ± 1.58[c,d/]	237.94 ± 1.41
5th[e/]	709.35 ± 1.22[d/]	445.67 ± 3.17
6th[e/]	694.52 ± 3.83[d/]	475.67 ± 3.37
	Crude Homogenates	
6th[e/]	237.33 ± 1.25	171.39 ± 1.38

a/ Larvae used in all assays were age synchronized.
b/ Newly molted
c/ Mean ± SE of at least three experiments, each assayed in duplicate.
d/ Value significantly different (< 0.05) from S strain.
e/ 1 day old
f/ 2 day old.

TABLE 6. Microsomal biphenyl 4-hydroxylase activity[a] in various instars of R and S fall armyworm larvae.

Larval Instar	Specific Activity (pmol min^{-1} $mg\ protein^{-1}$)	
	R	S
4th	663.76 ± 2.25[b,d]	177.28 ± 2.75
5th	959.23 ± 29.15[d]	548.27 ± 57.90
6th		
1 Day	768.89 ± 62.80[d]	386.26 ± 16.14
2 Day	1045.28 ± 62.70[d]	634.08 ± 11.91
3 Day	730.37 ± 28.78[c]	529.75 ± 33.06

a/ Larvae used in all assays were age synchronized.
b/ Mean ± SE of at least three experiments, each assayed in duplicate.
c/ Value significantly different (< 0.05) from S strain.
d/ Value significantly different ($p < 0.01$ from S strain.

found to have clear guts and to be preparing cells in the artificial diet for pupation.

The activities of microsomal biphenyl 4-hydroxylase of all R larval instars in Table 6 were greater than in the S strain by the following factors: 4th (3.74X), 5th (1.75X), one day-old 6th (2.09X), two day-old 6th (1.65X), three day-old 6th (1.38X). The activities of all larval instars in the R strain on a per mg protein basis were statistically different from the S strain at a probability of $P < 0.01$ except for three day old 6th instar which showed a significance probability of $P < 0.05$.

C. N-demethylase

The activity of microsomal N-demethylase of two- day-old sixth instar R and S fall armyworm larvae is summarized in Table 7. There are no differences in activity on a per mg protein basis; however, the S strain is significantly ($P < 0.01$) more active on a per midgut basis. The maximum difference (3.2-fold) was observed in the sixth instar of R and S larvae.

D. Cytochrome P-450

The results summarized in Table 8 show that there was no significant difference in the Cytochrome P-450 content between R and S strains, although the R strain appeared to be consistently higher than the S strain.

E. Glutathione S-transferase and Epoxide Hydrolase

From Table 9, it can be seen that there was no significant differences in the glutathione S-aryltransferase activity between the R and S strains. This is also true for the epoxide hydrolase activity (Table 10).

TABLE 7. Microsomal N-demethylase activity from sixth-instar R and S fall armyworm larvae[a/]

Strain	N-demethylase	
	pmol min^{-1} mg $protein^{-1}$	pmol min^{-1} $midgut^{-1}$
R	849.38 ± 59.42[b/]	39.96 ± 2.78
S	883.35 ± 141.62	129.70 ± 25.24[c/]

a/ Larvae used in all assays were age synchronized.
b/ Mean ± SE of at least three experiments, each assayed in duplicate.
c/ Value significantly different ($P < 0.01$) from R strain.

TABLE 8. Cytochrome P-450 activity from midgut microsomes of sixth-instar R and S fall armyworm larvae[a/]

Strain	pmol P-450 $mg\ protein^{-1}$	pmol P-450 $midgut^{-1}$
R	269.70 ± 12.40[b/]	17.97 ± 0.10
S	235.30 ± 6.60	11.53 ± 0.31

a/ Larvae used in all assays were age synchronized.
b/ Mean ± SE of at least three experiments, each assayed in duplicate.

TABLE 9. Glutathione S-aryltransferase activity of midgut soluble enzyme fraction from sixth instar R and S fall armyworm larvae.[a/]

Strain	Specific activity (nmol DCNB conjugated min^{-1} mg $protein^{-1}$)
R	22.73 ± 0.45[b/]
S	24.12 ± 0.30

a/ Larvae used in all assays were age synchronized.
b/ Mean ± SE of at least three experiments, each assayed in duplicate.

TABLE 10. Microsomal epoxide hydrolase activity in sixth instar R and S fall armyworm larvae.[a/]

Strain	Epoxide Hydrolase ($nmol\ min^{-1}\ mg\ protein^{-1}$)
R	33.92 ± 0.69[b/]
S	30.63 ± 0.77

a/ Larvae used in all assays were age synchronized.
b/ Mean ± SE of at least three experiments, each assayed in duplicate.

TABLE 11. General and carboxylesterase activities from crude homogenates of sixth instar R and S fall armyworm larvae.[a/]

Strain	nmol α-naphthol min^{-1} $mg\ protein^{-1}$		nmol α-naphthol min^{-1} $midgut^{-1}$	
	General esterase	Carboxyl esterase	General esterase	Carbaryl esterase
R	920.00 ± .007[b/]	202.10 ± 11.13	1540 ± .001	356.00 ± 22.63
S	1003.5 ± .02	217.75 ± 18.45	1036.5 ± 22.63	191.00 ± 2.10

a/ Larvae used in all assays were age synchronized.
b/ Mean ± SE of at least three experiments, each assayed in duplicate.

F. Esterase

The activities of general esterases and carboxylyesterase in the R and S strains are summarized in Table 11. The results show that there was no significant difference in the general esterase and carboxylesterase activities between the R and S strains when midgut crude homogenates were used as the enzyme source. However, the activity of general esterases from midgut microsomes are significantly higher in the S strain compared to the R strain (Table 12).

G. AChE Kinetics

AChE activity was not significantly different bewteen the R and S strains (Table 13). Studies of AChE enzyme kinetics (Fig. 9) show that the K_m values from both the R and S strains are not different toward ATC. Although the maximum reaction velocity (V_{max}) is different (R-V_{max} = 0.345 nmol min^{-1} my $protein^{-1}$; S-V_{max} = 0.208 nmol min^{-1} ng $protein^{-1}$) toward the hydrolysis of ATC, their substrate binding affinities, K_m, are the same (K_m = 38.46). Attempts to obtain an inhibition constant (K_i) failed because carbaryl is a poor yet reversible inhibitor of cholinesterase (Mount and Oehme 1981). Carbamates bind less tightly to cholinesterase as compared to most organophosphorous insecticides (Mount and Oehme 1981). At 10^{-7} M to 10^{-5} M concentrations, the inhibition rate showed a flat, nonlinear response after 15 minutes of incubation with carbaryl against moth head homogenate. A linear increase in carbaryl inhibition from 10^{-4} to 10^{-3} M concentrations is shown in Fig. 10, thus verifying that high carbaryl molar concentrations are required to inhibit AChE.

H. In vitro Carbaryl Metabolism

In vitro carbaryl metabolism studies showed that the R strain produced 5.4X more carbaryl oxidative metabolites than did the S strain

TABLE 12. General and Carboxylesterase activities from microsomes of sixth instar R and S fall armyworm larvae.

Strain	Specific activity (nmol α-naphthol min^{-1} mg $protein^{-1}$)	
	General esterase	Carboxylesterase
R	286.20 ± 11.15[a/]	196.23 ± 0.32
S	477.57 ± 6.88[c/]	261.73 ± 2.23

a/ Larvae used in all assays were age synchronized.
b/ Mean ± SE of at least three experiments, each assayed in duplicate.
c/ Value significantly different ($P < 0.05$) from R strain.

TABLE 13. Acetylcholinesterase activity from moth heads of 1 to 2 day old mixed population R and S fall armyworms[a/]

Strain	Specific activity (nmol ATC metabolized min^{-1} mg $protein^{1}$)
R	339.05 ± 4.93[b/]
S	253.21 ± 16.73

a/ Larvae used in all assays were age synchronized.
b/ Mean ± SE of at least three experiments, each assayed in duplicate.

Fig. 9. Lineweaver-Burke plot for the reactions of R and S fall armyworm moth head acetylcholinesterase with acetylthiocholine. V = product formed (nmol min^{-1} mg $protein^{-1}$); [ATC] = substrate concentration (mM).

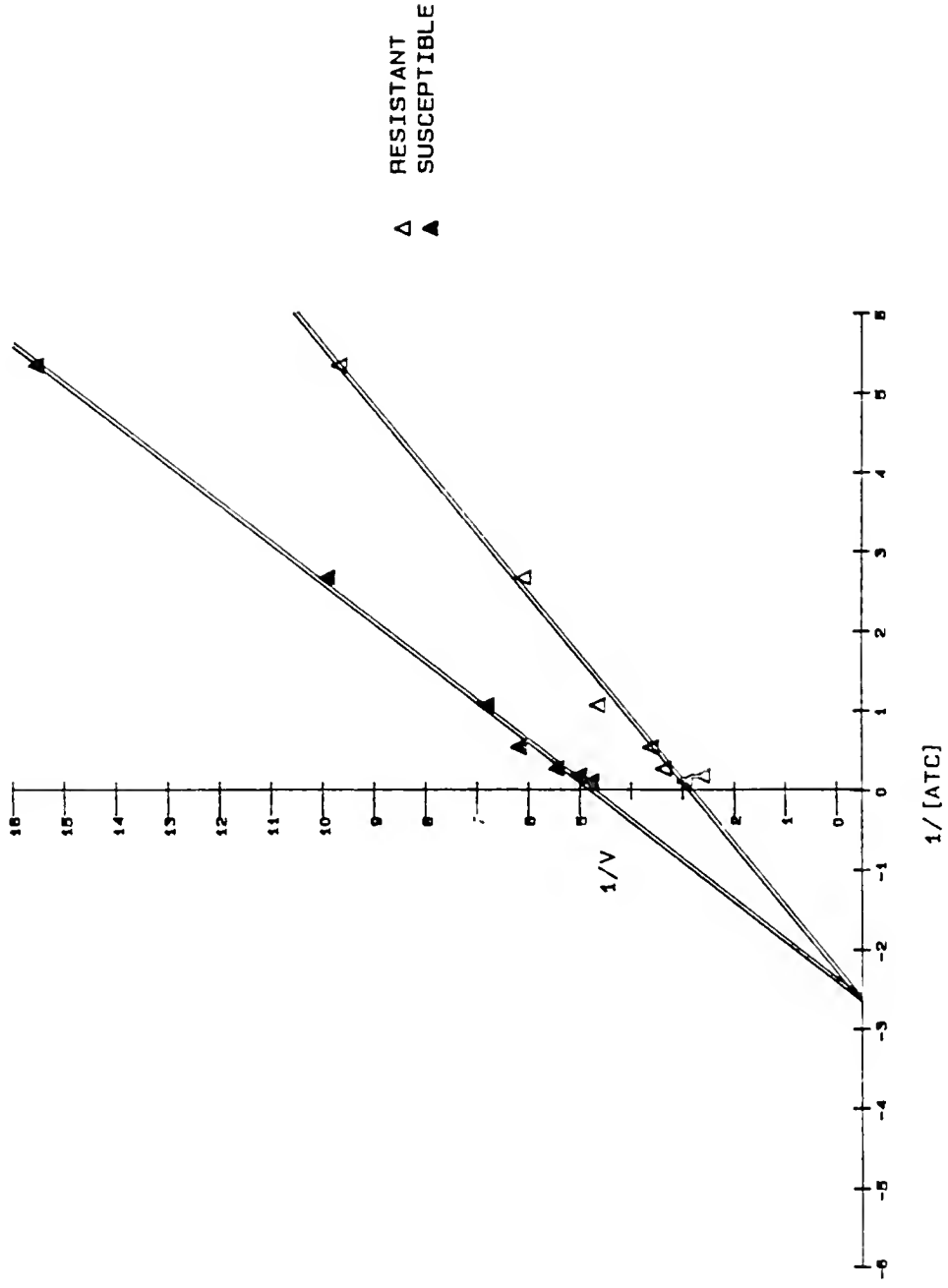

RESISTANT
SUSCEPTIBLE
1/V
1/[ATC]

Fig. 10. Carbaryl inhibition of AChE from heads of R and S fall armyworm adult moths.

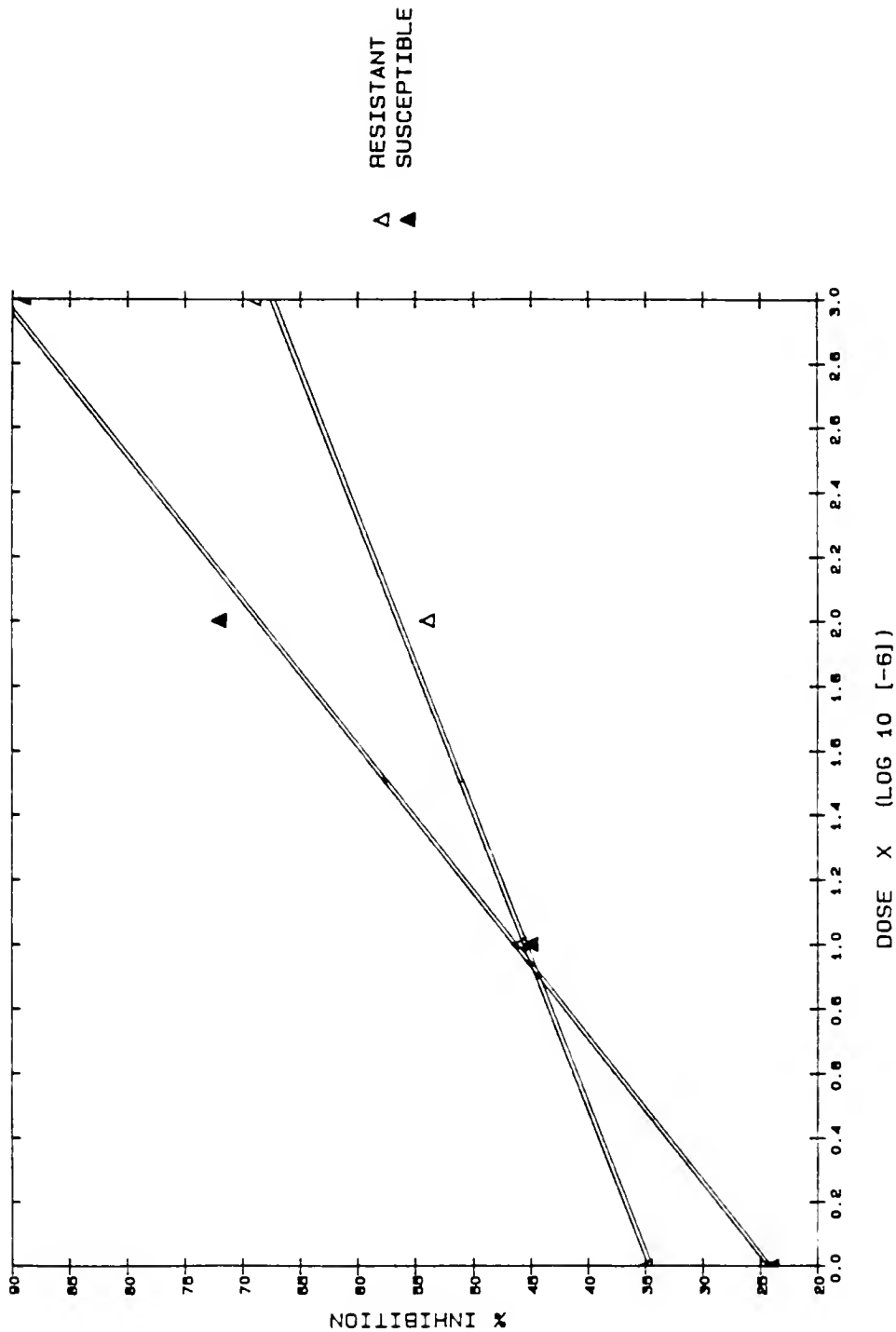

RESISTANT
SUSCEPTIBLE
% INHIBITION
DOSE X (LOG 10 [-6])

TABLE 14. *In vitro* metabolism of carbaryl by midgut homogenate from R and S fall armyworm larvae[a/]

Strain	Carbaryl metabolized (pmol 2 hr^{-1} mg $protein^{-1}$)				
	Esterase			Microsomal Oxidases	
	Control	TOCP (10^{-4} M)	DEF (10^{-4} M)	Control	PB (10^{-4} M)
R	567.45 ± 114.90	2.81 ± 3.67	0	168.72 ± 28.28	13.28 ± 2.86
S	578.53 ± 108.21	200.78 ± 15.01[b/]	159.68 ± 26.73[b/]	32.73 ± 4.32[b/]	13.26 ± 2.84

a/ Larvae used in this assay were 1-3 days old.
b/ Value significantly different ($P < 0.01$) from the R strain.

(Table 14). A 1.76X decrease in oxidative metabolites was seen when NADPH was not used in an incubation mixture. This suggests that oxidation depends on the NADPH cofactor for maximum reaction rate. The addition of the esterase inhibitors, DEF (10^{-4} M) and TOCP (10^{-4} M), to the incubation mixture reduced the esterase activity in the R strain by 99.5% and 100%, respectively. However, these inhibitors caused 65% and 72% reduction in esterase activity in the S strain suggesting that the S strain has a different form of esterase than the R strain. Similarly, microsomal oxidases which oxidized carbaryl were more susceptible to Pb inhibition in the R strain (92%) than in the S strain (59%).

The most significant data in the in vitro metabolism of carbaryl are the production of oxidative metabolites. The R strain metabolizes more carbaryl per unit time than does the S strain, thus confirming that oxidative metabolism plays a major role in resistance in this strain.

Cuticular Penetration

The rate of disappearance of [^{14}C] carbaryl from the exterior cuticle of sixth instar fall armyworm larvae is shown in Figure 11. After 24 hours, there remains almost 2X more carbaryl on the exterior cuticle of R larvae than of S strain. Figure 12 shows that the amount of carbaryl found internally in both R and S larvae is about the same at 13 and 14%, respectively, of the controls (immediate wash-off) (Fig. 12). Data in Figure 13 show that the S strain excretes more than 2X more carbaryl than the R strain in 24 hours, however. This suggests that more carbaryl enters S larvae and more is excreted either as carbaryl or as carbaryl metabolites while 60% of the applied carbaryl remains on the cuticle of R larvae.

Fig. 11. Percent of applied dose of [^{14}C] carbaryl remaining on the cuticle of sixth instar R and S fall armyworm larvae.

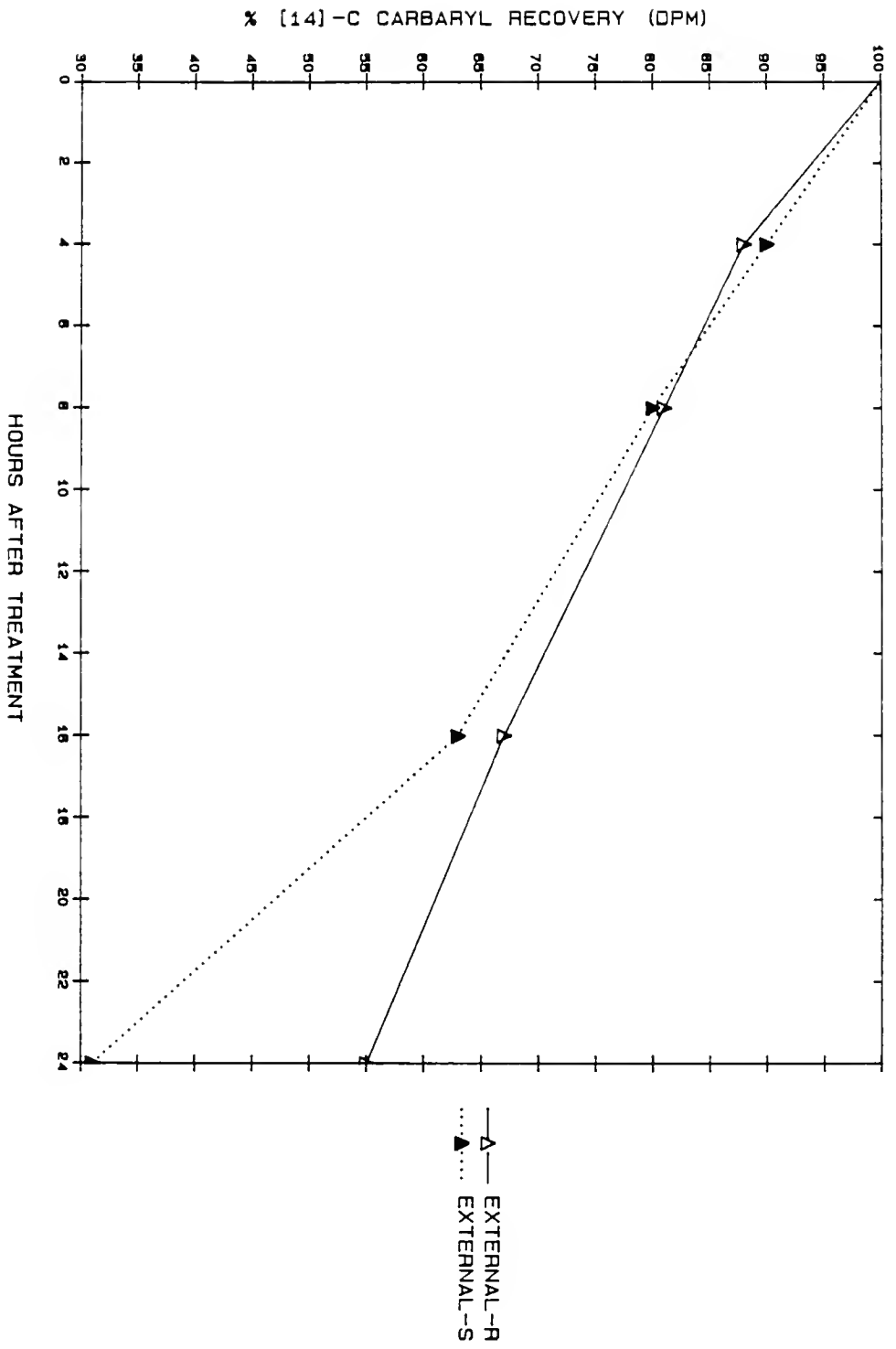

% [14]-C CARBARYL RECOVERY (DPM)
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
0
2
4
6
8
10
12
14
16
18
20
22
24
HOURS AFTER TREATMENT
EXTERNAL-R
EXTERNAL-S

Fig. 12. Percent of applied [^{14}C] Carbaryl extracted from homogenate of sixth instar R and S fall armyworm larvae.

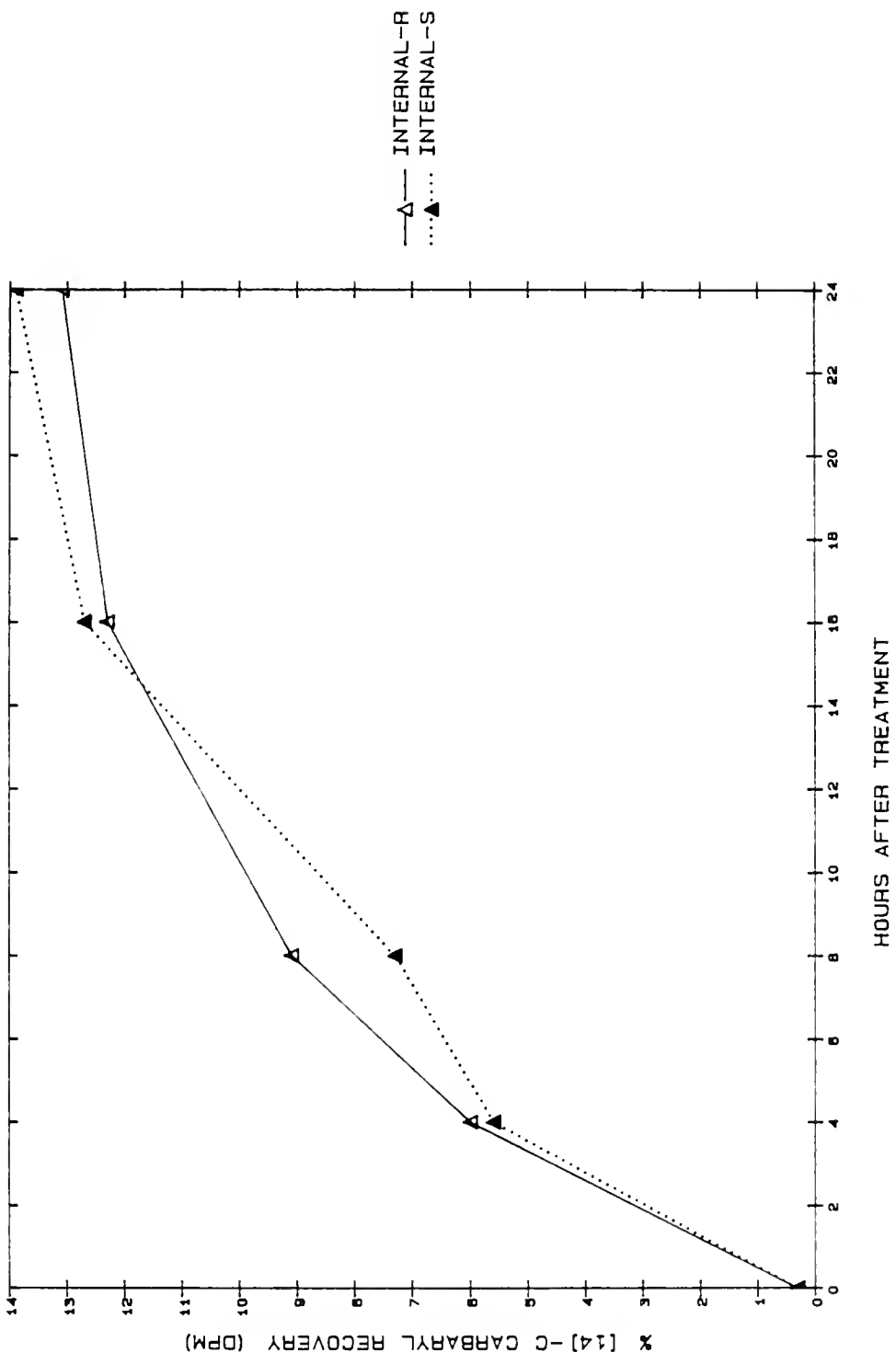
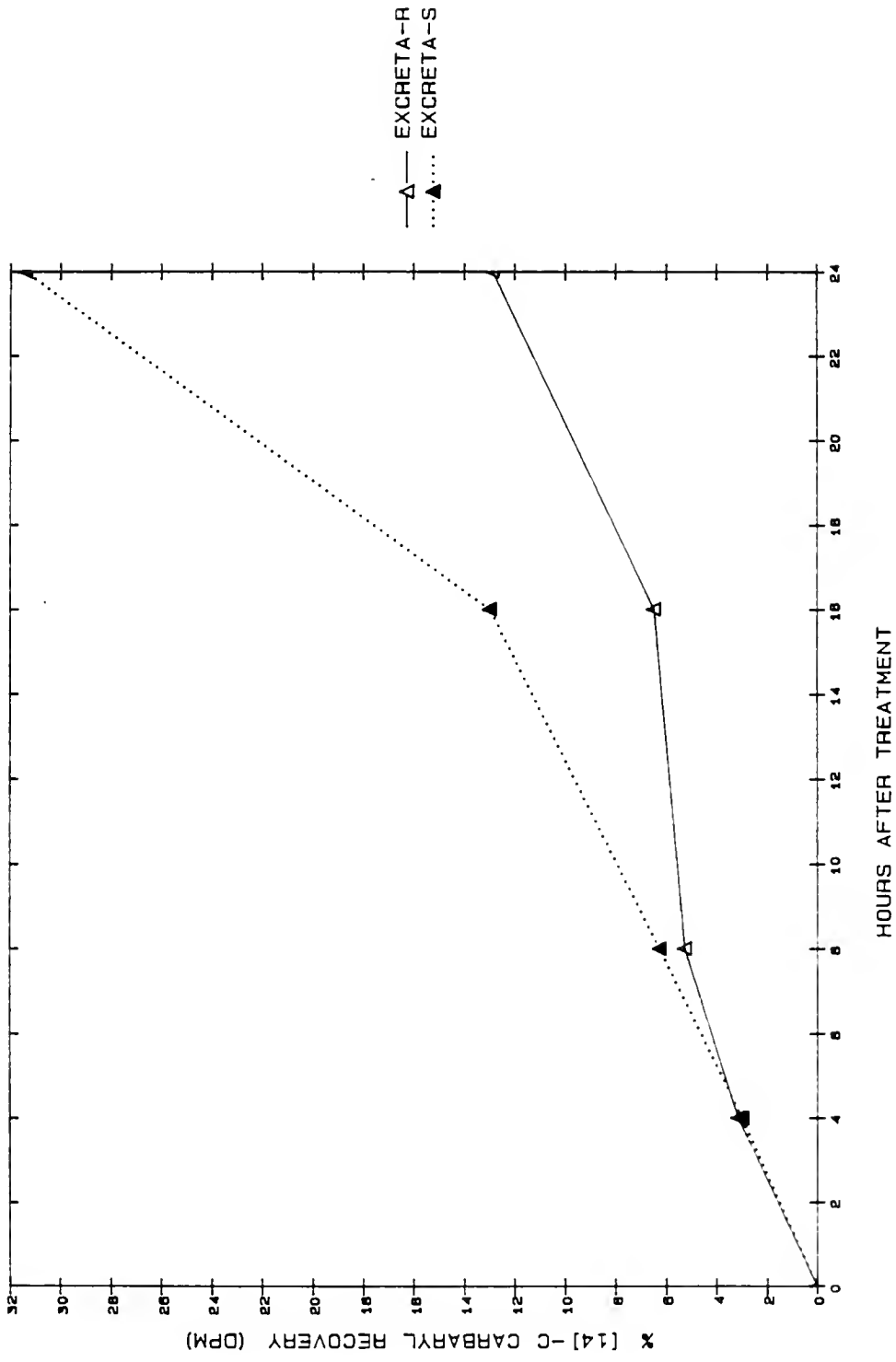

INTERNAL-R
INTERNAL-S
% [14]-C CARBARYL RECOVERY (DPM)
HOURS AFTER TREATMENT
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0
2
4
6
8
10
12
14
16
18
20
22
24

Fig. 13. Percent of applied ^{14}C-Carbaryl recovered from excreta of sixth instar R and S fall armyworm larvae.


EXCRETA-R
EXCRETA-S
% [14]-C CARBARYL RECOVERY (DPM)
HOURS AFTER TREATMENT
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

DISCUSSION

The results of this study show that the fall armyworm is resistant to carbaryl and confirms the findings of Young and McMillian (1979) and Wood et al. (1981). Young and McMillian (1979) also noted that R-FAW larvae were resistant to carbaryl biochemically and behaviorally. According to them, R-FAW larvae avoided carbaryl treated surfaces as compared to a carbaryl susceptible strain. Lockwood et al. (1984) cited this type of resistance as stimulus-dependent, i.e. requiring sensory stimulation to exhibit avoidance in this case, to avoid carbaryl residues. To further demonstrate the complexity of resistance in FAW, Wood et al. (1981) showed that FAW larvae that fed on previously carbaryl treated corn and signalgrass were resistant to carbaryl while those that fed on bermudagrass and millet were susceptible. These data agree with those of Yu (1984) where he found that midgut microsomes of a carbaryl resistant strain of FAW were highly induced by the allelochemicals, indole 3-carbinol and flavone.

In vivo data (Table 4) show that resistance could not be eliminated entirely by topical treatments of PB-carbaryl. These findings suggest that microsomal oxidases play a major role in resistance; however, there are other factors involved in resistance in this strain. Rose and Brindley (1985) showed that the Colorado potato beetle (CPB) was highly resistant to carbaryl. The topical treatment of these beetles with PB-carbaryl did not eliminate the resistance completely. They concluded that monooxygenases and other resistance mechanisms may be involved in

CPB resistance to carbaryl and carbofuran. This work agrees with my findings in FAW.

Results of in vitro assays show that the activities of aldrin epoxidase (Fig. 14) and biphenyl 4-hydroxylase (Fig. 15) are significantly higher in R-FAW larvae compared to S-FAW over all instars tested. Higher aldrin epoxidase and biphenyl 4-hydroxylase activities in R-FAW larvae were also observed by Yu (1984) and Yu and Ing (1984), respectively. These data further support in vivo findings that MFO enzymes play a major role in resistance in this strain.

In vitro metabolism of carbaryl in the R strain showed a 5-fold increase in oxidative metabolite production over the S strain (Table 14). The fact that differential inhibitions of carbaryl oxidation by PB were observed between R and S strains suggests that the MFO enzymes from the R strain were qualitatively different from the S strain. Kuhr (1971) and Kuhr and Davis (1975) identified carbaryl metabolites produced by midgut homogenates of R and S cabbage looper and European corn borer strains. They found that the oxidative metabolite, hydroxymethyl carbaryl, was the major metabolite produced in vivo and in vitro. Shrivastava et al. (1969) suggested that hydroxylation of substituted-aryl methylcarbamate toxicants contributed significantly to the development of resistance in a house fly strain. These findings are in agreement with my observations from the fall armyworm.

In the present study, carbaryl metabolites produced in vitro were not identified; however, those carbaryl metabolites that were found by other researchers (Price and Kuhr 1969; Camp and Arthur 1967; Andrawes and Dorough 1967; Kuhr 1970) were chromatographed by TLC and R_fs were recorded (Table 2). These R_fs were used to isolate carbaryl radiocarbons

Fig. 14. Aldrin epoxidase activities of midgut microsomes from various instars of R and S fall armyworm larvae.

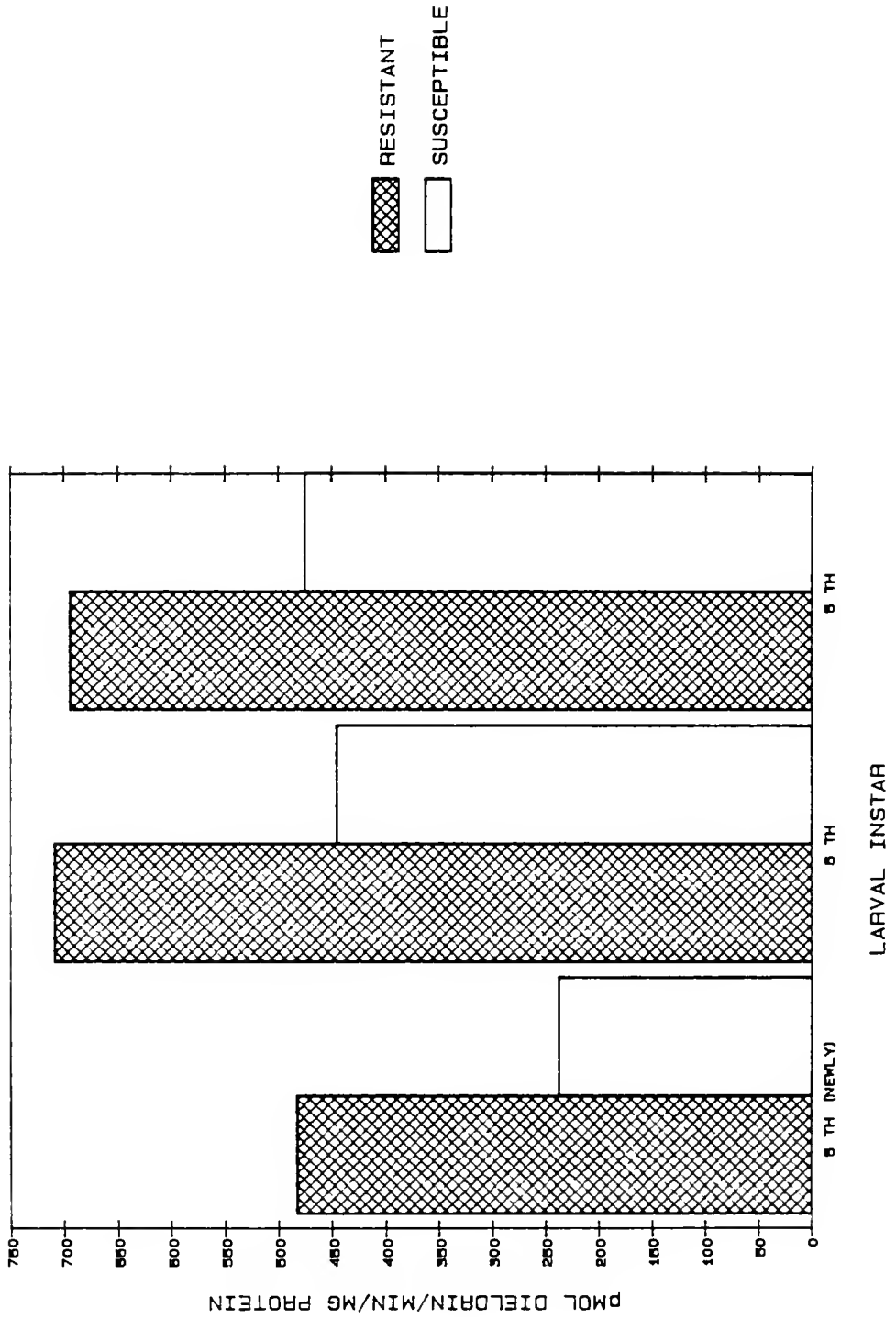
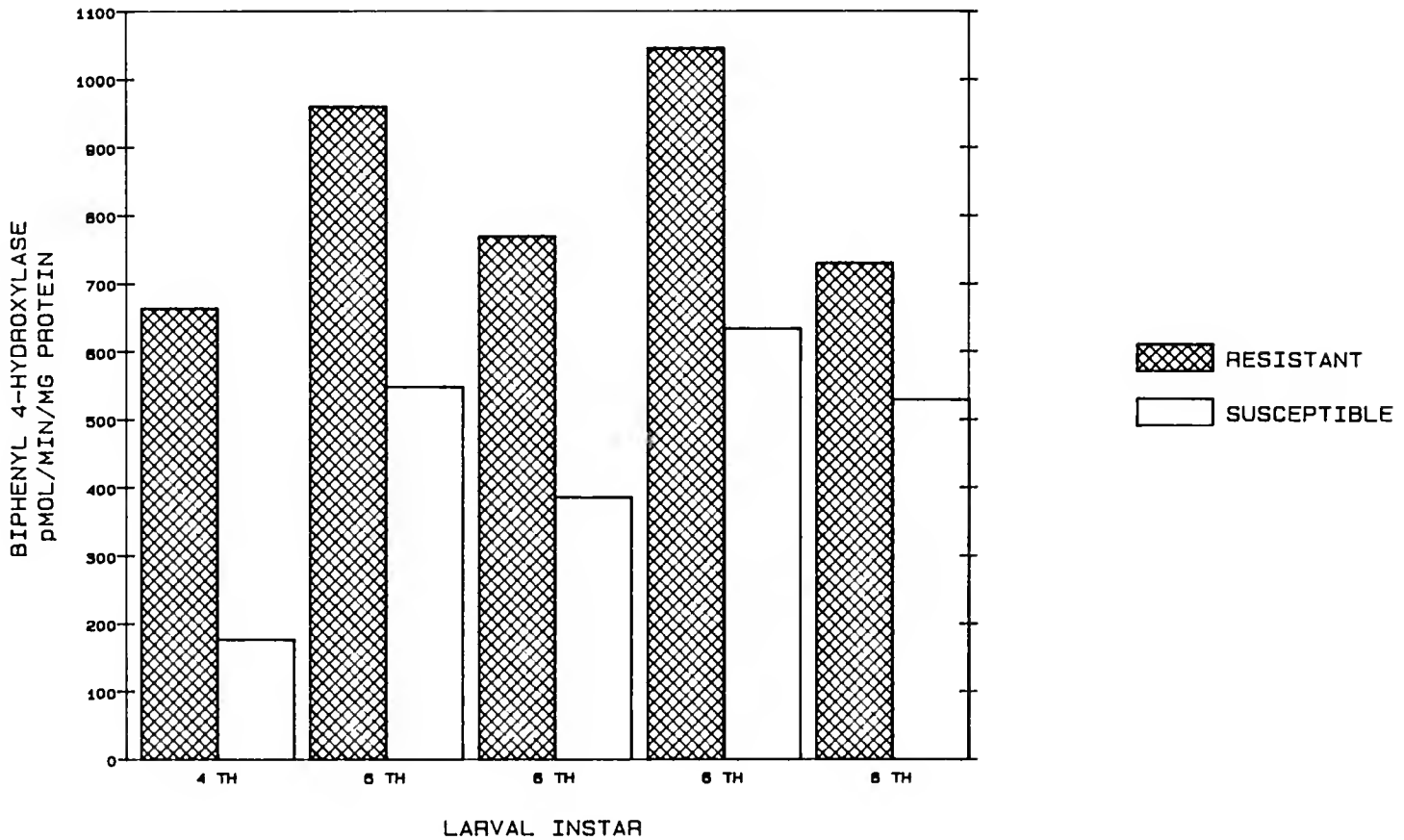

RESISTANT
SUSCEPTIBLE
pMOL DIELDRIN/MIN/MG PROTEIN
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
5 TH (NEWLY)
5 TH
5 TH
LARVAL INSTAR

Fig. 15. Microsomal biphenyl 4-hydroxylase activities from various instars of R and S fall armyworm larvae.



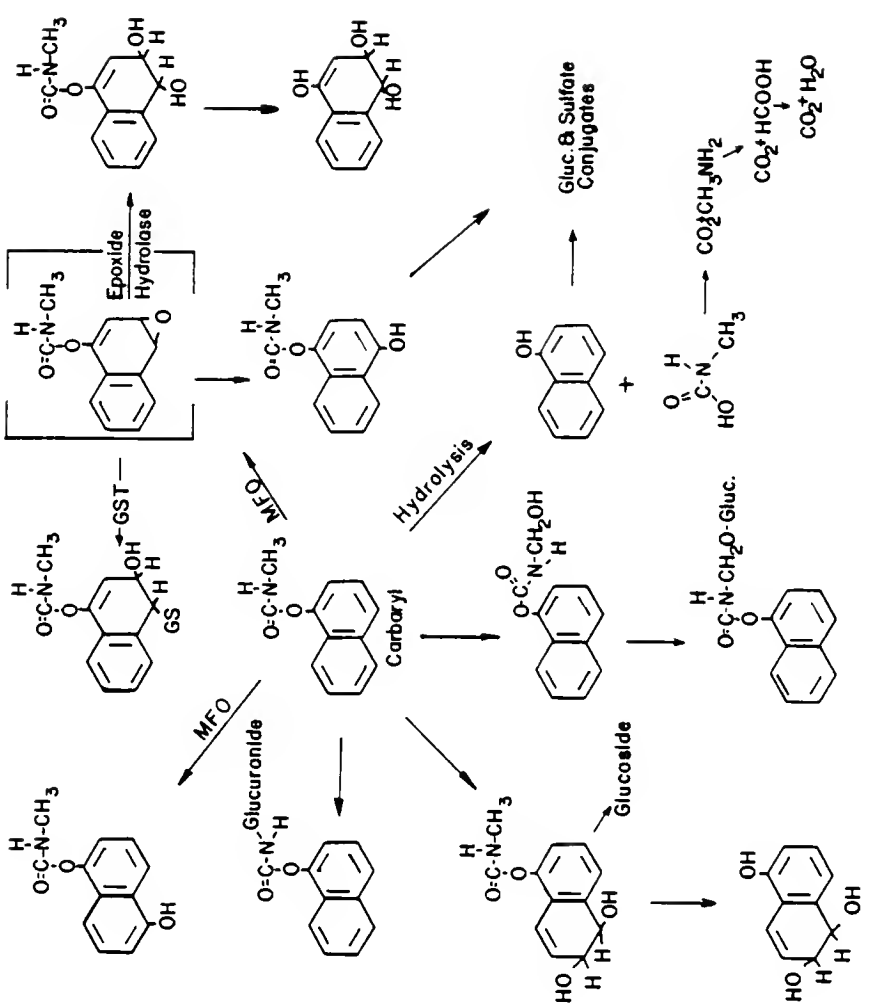
Groups of 6 th represent 1 day, 2 day and 3 day old larvae, respectively

by cochromatography on TLC plates and determine MFO activities. The metabolic pathways of carbaryl in various species (Menzie 1978) are summarized in Figure 16. Kuhr (1970) presented a partial carbaryl metabolic pathway which included the above metabolites. Andrawes and Dorough (1967) found hydrolytic and oxidative metabolism in the boll weevil and the boll worm similar to those in the present study. However, Andrawes and Dorough (1967) identified two additional metabolites from both insects as 1-hydroxy-5,6-dihydroxy-1-naphthyl N-methyl carbamate (see Fig. 16 for stuctures).

Additional radio-labelled compounds were found near the TLC plate origin but no attempts were made to identify them. Ahmad et al. (1980) and Andrawes and Dorough (1967) also found unknown radio-labelled compounds in this position; however, they made no attempts to identify them either.

The results obtained from carbaryl penetration studies are in agreement with those of Ariaratnam and Georghiou (1975) who found that carbaryl penetrated the cuticle of R Anopheles albimanus larvae about one half the rate of S larvae after 60 minutes. However, the difference was less apparent after 90 minutes. Another difference between their findings and mine, is that the mosquitoes were contained in an aqueous medium, in constant contact with the solublized insecticide. This factor may account for rapid absorption in the mosquito compared to FAW larvae. Hanna and Atallah (1971) described a consistant difference in the rate of carbaryl penetration into R and S Egyptian cotton leafworm larvae over a 72 hour period. These experiments show that Spodoptera spp. possess the ability for becoming resistant by decreased cuticular penetration.

Fig. 16. Metabolic pathways of carbaryl showing those enzyme systems that most likely produced them.


Epoxide Hydrolase
GST
MFO
Hydrolysis
Carbaryl
Gluc. & Sulfate Conjugates
Glucuronide
Glucoside
$CO_2CH_3NH_2$
CO_2 HCOOH
CO_2 H_2O

The amount of radiocarbon recovered from internal extracts (Fig. 12) of R and S larvae were similar; however, the amount of radiocarbon found in the excreta of S larvae was 2.4X higher than R larvae after 24 hours. These findings correspond well since the amount of carbaryl entering R larvae was less than in S larvae; therefore, there was less to excrete (Fig. 13). The levels of radiocarbon found in internal extracts of both strains probably represents sublethal levels that could be tolerated by larvae of both strains since no mortality was observed after 24 hours.

In summary, this study shows that the fall armyworm can become resistant to carbaryl via two mechanisms, increased metabolism and reduced cuticular penetration. The former appears to be the major factor and was likely to be caused by increased microsomal hydroxylation and epoxidation of carbaryl resulting in detoxication. Since behavioral mechanism (avoidance) was found to play a role in resistance, a combination of physiological as well as behavioral mechanisms may be responsible for carbaryl resistance in this insect.

In view of the findings presented here, the scope of carbaryl resistance in FAW would be completed if further research was performed on behavioral resistance mechanisms. Genetic investigations of inheritance, allelic contributions and chromosome location(s) of genes responsible for behavioral, metabolic and penetration resistance is also required. This information will provide researchers with greater knowledge of the complexities of insect resistance and contribute to the understanding of ways to circumvent it.

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BIOGRAPHICAL SKETCH

Elzie McCord, Jr., was born on May 6, 1949, in Vidalia, Georgia. He is the son of Elzie and Pearlene Calliberdena who have three other children; Euteria, Arlene LaVerne and Phyllis Olivia. Elzie attended public school at the James D. Dickerson High school where he earned the high school diploma with honors.

Elzie entered Savannah State College in 1967 to pursue a B.S. degree in biology with a minor in chemistry. He successfully completed that four year program, married the former Pinkie B. Wilmore, and immediately began postbacculaureate studies at the University of Florida with the assistance from a Rockefeller Grant. He was formally admitted to the graduate program of IFAS's (Institute of Food and Agricultural Sciences) Department of Entomology and Nematology in 1972 to pursue an M.S. degree with an emphasis on economic entomology.

Elzie received the M.S. degree in June of 1974 and accepted a one-year interim position with the Florida Cooperative Extension Service as Assistant in Extension Entomology. He was accepted to the doctoral program at the University of Florida in 1975; however, in June of 1976 he accepted a position as Biologist with the E. I. Du Pont de Nemours & Company, Wilmington, Delaware.

Elzie and Pinkie welcomed the birth of two sons, Rogers Christopher (2/1/77) and Timothy Ryan (7/9/78) who continue to provide them with many hours of happiness.

However, in January 1982, Elzie was granted a 18 months leave of absence from the Du Pont Company and his family to return to the doctoral program at the University of Florida. There he was fortunate

to work on carbaryl resistance in the fall armyworm under the direct supervision of Dr. Simon S. J. Yu.

Elzie completed the Ph.D. degree in Entomology specializing on August 10, 1985, and he returned to his Research Biologist's postion at Du Pont in Wilmington, Delaware. His area of specialization was insect toxicology.

Elzie McCord, Jr. is a member of the Entomological Society of America and the Florida Entomological Society.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

S. S. J. Yu, Chairman
Associate Professor of
Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

J. L. Nation
Professor of Entomology
and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

S. H. Kerr
Professor of Entomology
and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

R. B. Shireman
Associate Professor of
Food Science

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1985

Dean, College of Agriculture

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